

ABSTRACT

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JIAOGULAN AND HPLC FINGERPRINTING
FOR DIFFERENTIATION AND QUALITY
CONTROL

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To promote its use in nutraceutical and functional food ingredients, jiaogulan (*Gynostemma pentaphyllum*) of different sources, genotypes and parts of the plant were investigated for their chemical profiles and biological properties. The first part of this research studied five commercial jiaogulan for their phytochemical profiles, and antioxidant, anti-proliferative and anti-inflammatory effects. It was found that individual jiaogulan samples significantly differed in their chemical and biological properties. The second part of this research further investigated phytochemical compositions and biological activities in different genotypes and parts of jiaogulan. The results indicated that different genotypes or plant parts of jiaogulan may have different biological activities. The results led to the third study to develop chromatographic fingerprinting techniques for differentiating genotypes and plant parts of jiaogulan using LC/MS. LC-MS fingerprints combined with PCA were able to differentiate diploid and tetraploid, and the leaf and whole botanical jiaogulan. The

results from this research suggest the potential use of selected jiaogulan to improve human health while enhancing food agriculture economy, and indicate that HPLC fingerprinting may be a useful controlling the quality of jiaogulan.

NUTRACEUTICAL PROPERTIES OF JIAOGULAN AND
HPLC FINGERPRINTING FOR DIFFERENTIATION AND QUALITY CONTROL

By

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Table of Contents

Acknowledgements	ii
Table of Contents	iii
List of Tables	vi
List of Figures	vii
Introduction	1
Chapter 1: Literature Review	2
1.1 Distribution, Growing Conditions and Genetics of Jiaogulan	2
1.2 Bioactive Compounds in Jiaogulan	3
1.2.1 Polysaccharides	4
1.2.2 Flavonoids	5
1.2.3 Saponins	8
1.2.4 Carotenoids	10
1.2.5 Chlorophylls	10
1.2.6 Others (Sterols, lignan, Ge and Se)	11
1.3 General Health Benefits of Jiaogulan	14
1.3.1 Cardiovascular Disease	14
1.3.2 Diabetes	16
1.3.3 Cancer	17
1.3.4 Hepatic and Other Chronic Diseases	19
1.4 Inflammation, Immunity and Health	20
1.5 Reactive Species, Oxidative Stress and Health	23
1.6 Antioxidant and Health	25
1.7 Diploid and Tetraploid	27
1.8 HPLC/MS Fingerprinting	28
1.9 Regulation and Marketing	30
Chapter 2: Chemical Composition of Five Commercial <i>Gynostemma pentaphyllum</i> Samples and Their Radical Scavenging, Anti-proliferative, and Anti-inflammatory Properties	33
2.1 Abstract	33
2.2 Introduction	34
2.3 Materials and Methods	36
2.3.1 Materials	36
2.3.2 Sample Preparation	37
2.3.3 Determination of Water Content and Se Concentration	37
2.3.4 Total Fiber Content	37
2.3.5 Total Phenolic Content (TPC)	38
2.3.6 Total Saponin Content (TSC)	38
2.3.7 Total Flavonoid Content (TFC)	38
2.3.8 Flavonoid Profile	39
2.3.9 Relative DPPH Radical Scavenging Capacity (RDSC)	39
2.3.10 ESR Spin-Trapping Assay for DPPH Radical Scavenging Capacity ..	40
2.3.11 Hydroxyl Radical Scavenging Capacity (HOSC)	40
2.3.12 HT-29 Colon Cancer Cell Proliferation Inhibition	41

2.3.13	TNF- α , IL-6 and COX-2 Gene Expression Inhibition in Mouse J774A.1 Macrophage Cells	41
2.3.14	Statistic Analysis.....	42
2.4	Results and Discussion	43
2.4.1	Total Phenolic Content	43
2.4.2	Total Saponin Content	44
2.4.3	Total Flavonoid Content, and Rutin and Quercetin Concentrations	44
2.4.4	Relative DPPH Radical Scavenging Capacity (RDSC).....	48
2.4.5	Hydroxyl Radical Scavenging Capacity (HOSC).....	50
2.4.6	Effects of <i>G. pentaphyllum</i> Components on HT-29 Cell Proliferation ..	52
2.4.7	Effects of <i>G. pentaphyllum</i> Extracts on TNF- α , IL-6 and Ptg2 mRNA Expression.....	53
2.4.8	Water Content, Fiber Content and Selenium Concentration	56
Chapter 3: Chemical Composition of Five Commercial <i>Gynostemma pentaphyllum</i> Samples and Their Radical Scavenging, Anti-proliferative, and Anti-inflammatory Properties		58
3.1	Abstract	58
3.2	Introduction.....	59
3.3	Materials and Methods.....	61
3.3.1	Materials	61
3.3.2	Testing Sample Preparation	62
3.3.3	Determination of Se Concentration	62
3.3.4	Moisture Content	62
3.3.5	Fiber Content	62
3.3.6	Total Phenolic Content (TPC)	63
3.3.7	Total Flavonoid Content (TFC)	63
3.3.8	Total Saponin Content (TSC)	64
3.3.9	Rutin and Quercetin Contents.....	64
3.3.10	Relative DPPH Radical Scavenging Capacity (RDSC).....	65
3.3.11	Hydroxyl Radical Scavenging Capacity (HOSC).....	65
3.3.12	HT-29 Colon Cancer Cell Proliferation Inhibition	65
3.3.13	LNCaP Prostate Cancer Cell Proliferation Inhibition.....	66
3.3.14	Effects on TNF- α , IL-6 and COX-2 Gene Expression in Mouse J774A.1 Macrophage Cells.....	67
3.3.15	Statistic Analysis.....	68
3.4	Results and Discussion	68
3.4.1	Total Phenolic Content (TPC)	68
3.4.2	Total Flavonoid Content (TFC) and Rutin and Quercetin Contents.....	71
3.4.3	Total Saponin Content (TSC)	72
3.4.4	Relative DPPH Radical Scavenging Capacity (RDSC).....	73
3.4.5	Hydroxyl Radical Scavenging Capacity (HOSC).....	75
3.4.6	Effects of <i>G. pentaphyllum</i> Components on HT-29 Cell Proliferation ..	76
3.4.7	Effects of <i>G. pentaphyllum</i> Components on LNCaP Cell Proliferation .	78
3.4.8	Effects of <i>G. pentaphyllum</i> Extracts on TNF- α , IL-6 and Ptg2 mRNA expression in LPS-stimulated Mouse J774A.1 Macrophage Cells.....	81
3.4.9	Fiber Content, Selenium Concentration and Moisture Content.....	84

Chapter 4: Chromatographic fingerprint analysis, and rutin and quercetin compositions in the leaf and whole-plant samples of diploid and tetraploid <i>Gynostemma pentaphyllum</i>	86
4.1 Abstract	86
4.2 Introduction	87
4.3 Materials and Methods	89
4.3.1 Chemicals and Materials	89
4.3.2 Sample and Reference Preparation	90
4.3.3 Total Flavonoid Content	90
4.3.4 HPLC-DAD-MS Procedure	91
4.3.5 Data Analysis	92
4.4 Results and Discussion	92
4.4.1 Selection of the Extraction Condition	92
4.4.2 Rutin and Quercetin Contents	94
4.4.3 Validation of Chromatographic Fingerprinting Methodology	96
4.4.4 Similarity Calculation of the HPLC Fingerprint Analysis	100
4.4.5 Principal Components Analysis (PCA)	104
4.4.6 Chemical Markers	105
Summary	108
References	109

List of Tables

Table 1.1. Summary of Bioactive Compounds in <i>G. pentaphyllum</i> [*]	13
Table 2.1. Phytochemical Properties of <i>G. pentaphyllum</i> [*]	47
Table 2.2. Water Content, Fiber Content and Selenium Concentration of <i>G.pentaphyllum</i> [*]	56
Table 3.1. Phytochemical Properties and Moisture Content of <i>G. pentaphyllum</i> [*]	69
Table 3.2. Soluble and Insoluble Fiber Content and Selenium Concentration of <i>G.</i> <i>pentaphyllum</i> [*]	84
Table 4.1. Effects of Extraction Solvent and Method on Phytochemical Properties of <i>G. pentaphyllum</i> [*]	93
Table 4.2. Rutin and Quercetin Contents in <i>G. pentaphyllum</i> [*]	94
Table 4.3. MS Fragmentation of the Investigated Compounds by HPLC-MS [*]	99
Table 4.4. The Similarities of the Different <i>G. pentaphyllum</i> Samples.....	103

List of Figures

Figure 1.1. Basic Skeleton Structure and Common Types of Flavonoids.	5
Figure 1.2. Representative Structure of Gypenoside. $R_1 = \text{H}$, glucose, rhamnose; $R_2 = \text{H}$, glucose, rhamnose; $R_3 = \text{H}$, glucose, xylose.	8
Figure 2.1. Relative DPPH Radical Scavenging Capacity (RDSC) of <i>G. pentaphyllum</i> Extracts.	48
Figure 2.2. DPPH Radical Scavenging Properties of <i>G. pentaphyllum</i> Extracts Determined by ESR Method.	50
Figure 2.3. Hydroxyl Radical Scavenging Capacity (HOSC) of <i>G. pentaphyllum</i> Extracts.	51
Figure 2.4. Effects of <i>G. pentaphyllum</i> Extracts on HT-29 Cell Growth.	52
Figure 2.5. Effects of Ethanol Extracts of <i>G. pentaphyllum</i> on TNF- α mRNA Expressions in Mouse J774A.1 Macrophage Cells.....	54
Figure 2.6. Effects of Ethanol Extracts of <i>G. pentaphyllum</i> on IL-6 mRNA Expressions in Mouse J774A.1 Macrophage Cells.....	55
Figure 2.7. Effects of Ethanol Extracts of <i>G. pentaphyllum</i> on Ptgs2 mRNA Expressions in Mouse J774A.1 Macrophage Cells.....	55
Figure 3.1. Relative DPPH Radical Scavenging Capacity (RDSC) of <i>G. pentaphyllum</i> Extracts.	75
Figure 3.2. Hydroxyl Radical Scavenging Capacity (HOSC) of <i>G. pentaphyllum</i> Extracts.	76
Figure 3.3. Effects of <i>G. pentaphyllum</i> Extracts on HT-29 Colon Cancer Cell Growth.	78
Figure 3.4. Effects of <i>G. pentaphyllum</i> Extracts on LNCaP Prostate Cancer Cell Growth.	80
Figure 3.7. Effects of <i>G. pentaphyllum</i> on Ptgs2 mRNA Expressions in Mouse J774A.1 Macrophage Cells.	83
Figure 4.1. HPLC Fingerprint of the Representative <i>G. pentaphyllum</i> Samples.....	100
Figure 4.2. Scores plot of PCA of <i>G. pentaphyllum</i> samples.	104
Figure 4.3. Loading plot of PCA.	106

Introduction

Jiaogulan (*Gynostemma pentaphyllum* Makino) is a botanical material traditionally used in food and tea. It is easy to grow in poor soil and is indigenous to China, Korea, Japan, Thailand and Vietnam. Growing evidence has revealed that jiaogulan may potentially reduce the risk of cardiovascular diseases, and may have hypoglycemic, anti-inflammatory, anticancer and hepatoprotective activities with little concern of toxic effects. However, most of the studies lack information on their growing locations, genotypes, or plant parts. There is no information about how different the commercial products could be. The quality of nutraceuticals can vary because of these factors.

The goal of this research was to improve the potential use of selected jiaogulan to improve human health while enhancing food and agriculture economy. The specific objectives were:

- 1) To examine the five commercial jiaogulan samples for their phytochemical profiles, and antioxidant, anti-proliferative and anti-inflammatory effects.
- 2) To investigate phytochemical compositions, and antioxidant, anti-proliferative and anti-inflammatory effects in different genotypes and plant parts of jiaogulan.
- 3) To investigate the chromatographic fingerprint in different genotypes and plant parts of jiaogulan by LC/MS.

Chapter 1: Literature Review

1.1 Distribution, Growing Conditions and Genetics of Jiaogulan

Jiaogulan (*Gynostemma pentaphyllum*) is a well-known botanical in Asian countries. It is also called “herb of immortality”, “southern ginseng”, Panta tea and Penta tea. It has long been consumed as energizing tea or food. The first use of jiaogulan can be traced back to 1406 CE in China as a survival food.

Jiaogulan is indigenous over large areas in Asia, e.g. Japan, China, Korea, India, Thailand, Malaysia and the Philippines. In China, jiaogulan is mostly produced in southwest provinces. It has been introduced into the US and is being sold in many companies as a single herb, in teas or in multi-herb formulations (Blumert & Liu, 1999).

Jiaogulan refers to a plant of the genus *Gynostemma*. There are over thirty species of *Gynostemma* in China. The species *G. pentaphyllum* has the widest distribution and is the most studied, and is the focus of this research. In the remainder of the text, jiaogulan and *Gynostemma pentaphyllum* will be used interchangeably. Jiaogulan is a perennial creeping vine that tolerates a wide-range of soil, temperature and light factors. It's distributed in mountains and plains between 1000-10000 feet (Blumert et al., 1999). A soil suitable for its cultivation should be aerated, rich in humus, nitrogen, phosphorus and moisture, and capable of holding water. The pH should be within 5.5-8.0 (optimal 6.5-7.0). Jiaogulan prefers a warm and humid environment. The viable weather temperature is -15 - 41.5 °C (optimal 15-30 °C). The viable relative illumination of the plant is between 40% and 80% (Optimal 65-75%)

(Razmovski-Naumovski, Huang, Tran, Li, Duke, & Roufogalis, 2005). With optimal condition, the herb can be harvested every 20-30 days with 4,000-5,000 kg of dried herb collected (Razmovski-Naumovski et al., 2005). The stem, leaf, root or the whole plant of jiaogulan has been used in food, traditional medicine and supplement products.

Ploidy is the number of sets of chromosomes in the nucleus of cells. Diploid cells have two sets of homologous chromosomes and polyploid refers to cells that have more than two sets of chromosomes. In most organisms, diploid is the majority. In plant, however, it's not uncommon to see polyploidy from natural selection as well as artificial breeding. The chromosomal numbers of jiaogulan are usually $2n = 22, 44, 66$ and 88 , indicating there are diploid, tetraploid, hexaploid and octoploids in jiaogulan plant. The hexaploids and octoploids are usually slightly larger than diploids and tetraploids in most plants. However, the diploid and polyploids of jiaogulan are usually indistinguishable from morphological observation (Jiang, Qian, Guo, Wang, & Zhao, 2009).

1.2 Bioactive Compounds in Jiaogulan

Jiaogulan contains a variety of nutrients and bioactive compounds including several types of polysaccharides, flavonoids, saponins, carotenoids, chlorophylls, sterols, amino acids, vitamins and minerals (Huang, Hung, Wu, & Chen, 2008a; Razmovski-Naumovski et al., 2005). Saponins (gypenosides) are thought to be the prominent group of bioactives that contribute to the health beneficial effects of jiaogulan.

1.2.1 Polysaccharides

Polysaccharides belong to carbohydrates, most of which consist of 100-3000 glycosyl units in linear or branched arrangements. Besides nutritional function, polysaccharides are believed to play an important bioactive role in botanical materials, such as immunomodulatory, antioxidant and anti-cancer effects. A number of polysaccharides that have been isolated and characterized from jiaogulan. Wang et al (2007) reported three polysaccharides (GMA, GMB & GMC) that were extracted from jiaogulan with water and fractionated by anion exchange chromatography. The major sugar constituent of GMA is glucose; GMB is a heteropolysaccharide with 36% of glucose, 36% of galactose and 28% of mannose; GMC is composed of 18% glucose, 40% galactose, 23% mannose and 19% fructose (Wang & Luo, 2007). Yang and others (2008) found a non-starch polysaccharide after water extraction and deproteination. It is a protein-bound polysaccharide that consists of xylose (3.9%), arabinose (10.5%), glucose (23.2%), rhamnose (7.7%), galactose (18.9%), mannose (3.1%), glucuronic acid (1.2%), and galacturonic acid (4.7%). Chi et al (2008) was able to extract three water soluble polysaccharides (GPP1-a, GPP2-b & GPP3-a) from jiaogulan. GPP1-a consists of 9% arabinose, 38% galactose and 53% glucose. The backbone of GPP1-a is (1→4)-linked Glucose with three kinds of branches. GPP2-b consists of 30% xylose, 39% galactose and 31% glucose. GPP3-a is composed of 44% ribose, 15% fructose, 13% galactose and 27% glucose. Lv and colleagues (2009) extracted another water soluble polysaccharide composed of mannose (4%), ribose (2%), rhamnose (11%), glucuronic acid (1%), galacturonic acid (6%), glucose (30%), xylose (6%), galactose (24%) and arabinose (17%), although the purity was not

verified. It is noteworthy that even with similar extraction procedure, jiaogulan (*G. pentaphyllum*) from different sources have very different reported polysaccharide profiles. A research group isolated a polysaccharide (GPP2) composed of rhamnose and xylose (1:12.25 molar ratio) with sulfated modifications. Sulfation of GPP2 increased the anti-tumor activity significantly (Chen, Li, Li, Zhao, Shen, & Zhang, 2011). The detailed health effects are discussed below in subsequent sections.

1.2.2 Flavonoids

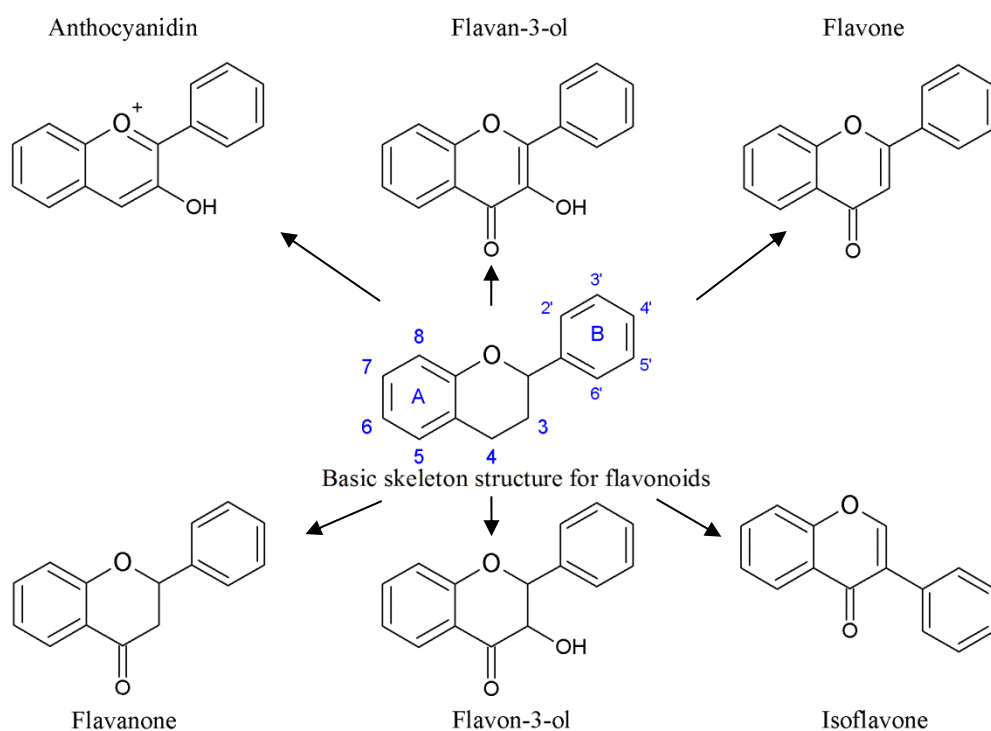


Figure 1.1. Basic Skeleton Structure and Common Types of Flavonoids.

Flavonoids belong to the larger group of phenolic compounds, which are widely present in plants. The basic structure of flavonoids consists of two aromatic rings, linked by a three-carbon bridge (**Figure 1.1**) (Brusselmans, Vrolix, Verhoeven, & Swinnen, 2005). According to the functional group and oxidation state, flavonoids

can be further classified into six subclasses: flavones, flavanones, flavanols, flavonols, isoflavones, and anthocyanidins (**Figure 1.1**). In nature, more than 6,500 flavonoids have been characterized. In plant, they are mostly in glycoside form, in which phenolic hydrogen(s) is/are substituted by a sugar moiety. Flavonoids are one of the most and abundant bioactive components found in jiaogulan (Kao, Huang, Inbaraj, & Chen, 2008). To date, quercetin-di-(rhamno)-hexoside, quercetin-rhamno-hexoside, kaempferol-rhamno-hexoside, rutin, kaempferol-rhamno-hexoside and kaempferol-3-O-rutinoside have been identified from jiaogulan (Kao et al., 2008; Tsai, Lin, & Chen, 2010a).

The flavonoid content of plants may vary greatly due to genotype, environment and also the combination effect of the two (Marotti & Piccaglia, 2006; Moore, Liu, Zhou, & Yu, 2006a; Whent, Hao, Slavin, Zhou, Song, Kenworthy et al., 2009). Kao and others reported a flavonoid content around 45 mg/g of jiaogulan (Kao et al., 2008). In another study the flavonoid content of jiaogulan was 123 mg/g of dry botanical (Tsai et al., 2010a). These values represent jiaogulan samples procured in Taiwan. Yet it is possible that jiaogulan of different genotypes from other areas might have dissimilar values.

It's also known that solvent selection may alter the extraction efficiency of flavonoids. Due to the structure variations, it may not be possible to find an optimal solvent capable of extracting all flavonoids. Aqueous methanol containing HCl has frequently been used to extract and hydrolyze flavonoids from fruits and vegetables (Chu, Chang, & Hsu, 2000; Crozier, Lean, McDonald, & Black, 1997; Hertog, Hollman, & Katan, 1992; Miesan & Mohamed, 2001). Marotti and Piccaglia (2006)

utilized methanol/water/acetic acid to extract flavonoids from onion. Compared to methanol, ethanol, water and ethanol/water, the methanol/water mixture was shown to give the highest yield of flavonoids in weld (*Reseda luteola* L.) (Cristea, Bareau, & Vilarem, 2003).

Other factors influencing the yield of flavonoid extraction include extraction temperature, extraction time, plant species and target part of plant. Cristea and others found 2 h extraction time with boiling temperature can achieve the best efficiency in flavonoid extraction (Cristea et al., 2003). Plant species is also an important factor in yield of flavonoid extraction (Cho, Howard, Prior, & Clark, 2004). In addition, flavonoids are usually distributed unevenly into different plant parts (Romani, Vignolini, Galardi, Aroldi, Vazzana, & Heimler, 2003).

The amount of total flavonoids is typically measured by colorimetric methods. The most common one is the aluminum chloride colorimetric method. In this method, aluminum chloride binds to C-4 keto group and either the C-3 or C-5 hydroxyl group of flavonoids to form acid-stable complexes. The complex showed strong absorbance between 385 nm to 440 nm corresponding to the concentration of flavonoids (Chang, Yang, Wen, & Chern, 2002). Another method involves 2,4-dinitrophenyl-hydrazine. It reacts with ketones and aldehydes in flavonoids to form 2,4-dinitrophenyl-hydrazones with an maximal absorbance of 495 nm (Chang et al., 2002). For individual flavonoid profile investigation, HPLC is the predominate method used (Chen, Ozcan, & Harnly, 2007; Tomás-Barberán, Martos, Ferreres, Radovic, & Anklam, 2001). This procedure generally uses reverse phase HPLC column with an appropriate water/organic solvent system for elution. Flavonoids can be detected at a

specific UV absorbance range. Recently, mass spectrometry has also been implemented for higher accuracy measurement (Kao et al., 2008).

1.2.3 Saponins

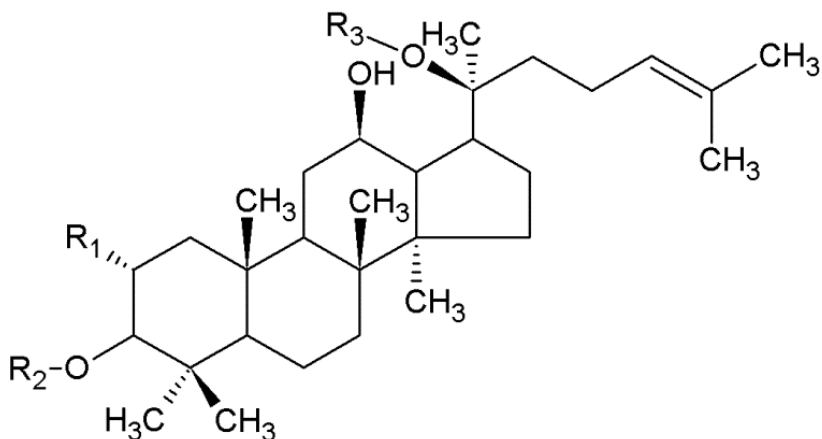


Figure 1.2. Representative Structure of Gypenoside. R_1 = H, glucose, rhamnose; R_2 = H, glucose, rhamnose; R_3 = H, glucose, xylose.

Saponins are a group of compounds widely distributed in plants. They are mainly composed of sapogenin (aglycones), hexoses and galacturonic acid (**Figure 1.2**). There are three types of saponins: steroidal saponins, *N*-containing steroidal-saponins and triterpenoid saponins. The best known saponins are ginsenosides in ginseng. They are thought to have adaptagenic properties (Blumert et al., 1999). Saponins in jiaogulan are mainly triterpenoid saponins (dammarane type). They are considered the main health beneficial compounds in jiaogulan, named gypenosides or gynosaponins (Kao et al., 2008). In gypenosides, the main sugar types are β -D-glucose, β -D-xylose, α -L-arabinose and α -L-rhamnose in positions C-3 (β) and C-20. The functional groups are hydroxyl, methyl, aldehyde, alcohol and the less common, ketone in position C-19; a hydroxyl group in position C-2 (α) and C-12 (β) (Razmovski-Naumovski et al., 2005); and an epoxy ring in position C-17 (Liu, Ye, Mo, Yu, Zhao, Wu et al., 2004b) (**Figure 1.2**). Eight gypenosides have the same

structures as ginsenoside Rb1, Rc, Rb3, Rd, F2, Rg3, malonyl-Rb1, malonyl-Rd and Rf, which were once considered unique in ginseng (Razmovski-Naumovski et al., 2005). Since jiaogulan is a vine botanical widely grown over Asia, the saponins in jiaogulan have been the subject of recent research interest.

Saponins in jiaogulan are reported to be responsible for the effects on lipid metabolism, cardiovascular system, blood sugar control, neural system, cancer prevention and treatment, inflammation, as well as anti-fatigue activities (Razmovski-Naumovski et al., 2005). The detailed health beneficial effects will be discussed in subsequent sections. The total saponin content of jiaogulan varies by species, growing locations and collection time (Razmovski-Naumovski et al., 2005; Yin & Hu, 2006). Over 170 saponins have been discovered in jiaogulan to date (Kim & Han, 2011). Generally it is reported to be about 2.4% of the dry weight of the plant (Razmovski-Naumovski et al., 2005). The structural similarities of gypenosides complicate the methods of isolation for individual gypenosides (Razmovski-Naumovski et al., 2005). It typically involves solvent extraction, followed by different types of chromatographic separations. Due to the focus of this proposal, the detailed information of the techniques of isolation would not be discussed in this review.

Colorimetric and chromatographic methods are widely used for determination of saponins. Steroidal sapogenins with or without double bond at C-5, triterpenoid sapogenins, sterols and bile acids which have an OH group at their C-3 position, reacting with vanillin and sulfuric acid, could produce an absorbance at 460-485 nm (Hiai, Oura, & Nakajima, 1976). Chromatographic methods are also used for saponin determination. Due to the absence of chromophores in saponins, HPLC coupled with

ELSD or MS detectors are used to detect saponins with individual reference standards (Kao et al., 2008).

1.2.4 Carotenoids

Carotenoids are a group of organic pigments and antioxidants that exclusively exist in plants. They absorb blue light in general. Carotenoids are tetraterpenoid with two major types: xanthophylls which contain oxygen, and carotenes which are purely hydrocarbons. Due to their conjugated structures, carotenoids are vulnerable to light, heat and oxygen degradation. Three carotenoids, α -carotene, β -carotene, and β -cryptoxanthin, are considered the precursors of Vitamin A. Carotenoids have been reported in jiaogulan of different varieties (Liu, Kao, & Chen, 2004a). The highest concentration of carotenoids found in jiaogulan is all-trans-lutein (72-377 $\mu\text{g/g}$ dry material), followed by 13-cis-lutein (30-73 $\mu\text{g/g}$) (Liu et al., 2004a). The high content of all-trans-lutein and cis-lutein may contribute to jiaogulan's antioxidant, anti-inflammatory and cardiovascular disease prevention properties (Liu et al., 2004a).

Carotenoids can be measured by chromatographic methods. Typical condition includes reverse-phase C18 or C30 columns for separation, with a diode array detector (Liu et al., 2004a; Oliver & Palou, 2000).

1.2.5 Chlorophylls

Chlorophylls are green pigments found in almost all plants. They are important in photosynthesis processes of plants. They are also thought to have several health effects in humans such as antioxidant activity (Lanfer-Marquez, Barros, &

Sinnecker, 2005), immunomodification (Sharma, Kumar, & Sainis, 2007), anti-cancer (Nakamura, Murakami, Koshimizu, & Ohigashi, 1996), anti-inflammatory effect (Nakamura et al., 1996) and antigenotoxic effect (Bez, Jordão, Vicentini, & Mantovani, 2001). The basic structure of chlorophylls includes a porphyrine with a magnesium ion in the center and a tail which is a phytol group. In general, two types of chlorophylls, chlorophyll A and chlorophyll B are present in plant in a ratio of 3:1 (mole:mole). A number of derivatives exist in plants. In jiaogulan, 15 chlorophylls and their derivatives have been separated and identified, as reported by a recent study (Huang et al., 2008a). This study found the predominant chlorophyll in jiaogulan as pheophytin A (2508.3 µg/g), followed by pheophytin B (319.6 µg/g) and chlorophyll B (287.6 µg/g). It is noteworthy to mention that the content of chlorophylls and their derivatives may change due to degradation and conversion under light, heat, acidic and enzymatic conditions (Huang et al., 2008a).

Eighty percent acetone is often used for extraction of chlorophylls. For accurate determination of chlorophylls and their derivatives, reverse phase HPLC or even liquid chromatography–mass spectrometry (LC-MS) is usually implemented (Huang et al., 2008a; Schwartz, 1998).

1.2.6 Others (Sterols, lignan, Ge and Se)

Other than the nutrients mentioned above, there are several other important phytochemicals reported in jiaogulan. Sterols include 24β/R-ethyl-5α-cholesta-7,22E-dien-3β-ol, 24,24-dimethyl-5α-cholest-7-en-3β-ol, 24,24-dimethyl-5α-cholesta-7,22E-dien-3β-ol, 24-methylene-14α-methyl-5α-cholest-9(11)-en-3β-ol, 24,24-dimethyl-5α-cholestan-3β-ol and 24α/R-ethyl-5α-cholestan-3β-ol (Akihisa, Mihara,

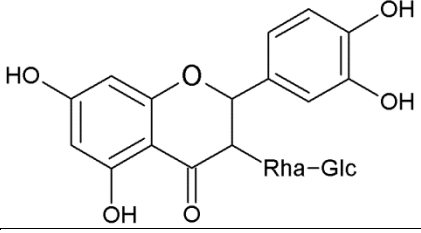
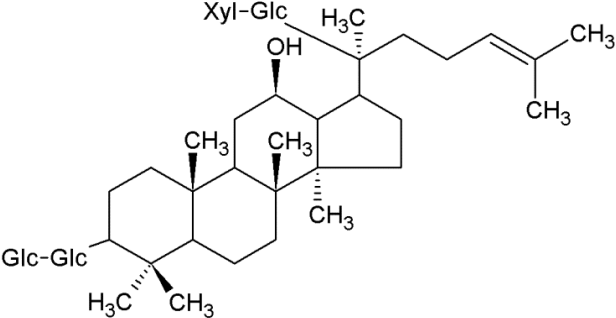
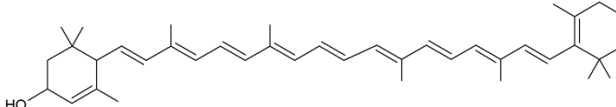
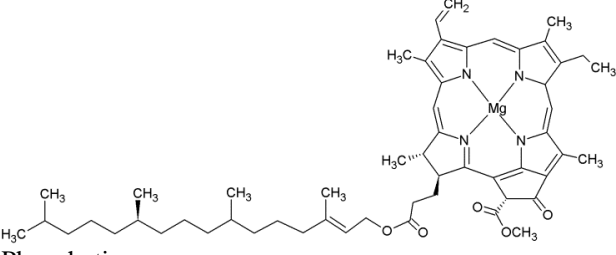
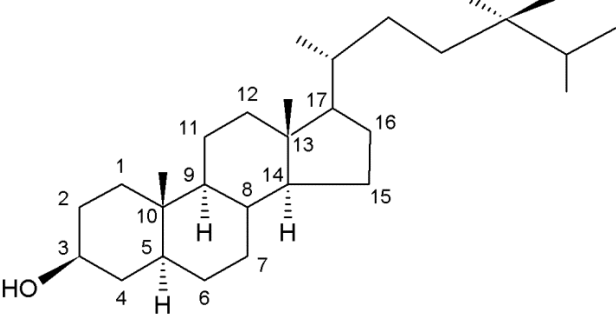
Fujikawa, Tamura, & Matsumoto, 1988). No studies have reports of sterols in jiaogulan on the biological properties.

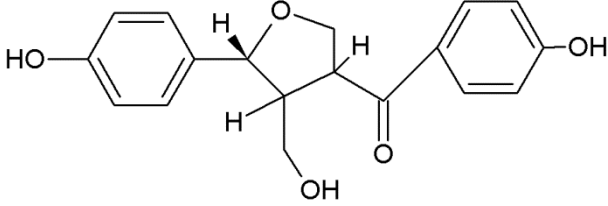
Lignans are one type of the major phytoestrogens. It has been reported that lignans be involved in cancer prevention and estrogen mechanism (Adlercreutz, 1998). The lignans found in jiaogulan are ligballinone 1 and ligballinol 2 (Wang, Zhang, Chen, Wang, & Wen, 2009). Ligballinone 1 was shown to exhibit antimicrobial activity yet the details have not been reported.

Trace elements are involved in vital enzymatic reactions in vivo. Trace elements in jiaogulan have been studied by Liang et al (2006). Ge (0.56 µg/g), Cu (8.81 µg/g), Zn (24.0 µg/g), Fe (454.7 µg/g) and Mn (78.7 µg/g) have been reported in dry preparations of jiaogulan (Liang & Sun, 2002).

The summary of the bioactive compounds in jiaogulan is shown below in **Table 1.1**.

Table 1.1. Summary of Bioactive Compounds in *G. pentaphyllum**

	Amount	Representative Compound	Ref
Polysaccharides	113-128 mg/g	GMA: Mw: 9.4×10^4 Composed of Glucose and Fructose (11.45:1 in mol)	Wang et al., 2007a, 2007b; Chi et al., 2008
Flavonoids	45-123 mg/g		Kao et al., 2008
Saponins	~24 mg/g	 Gypenoside IV (Ginsenoside Rb3)	Razmovski-Naumovski et al., 2005
Carotenoids	443-1127 µg/g	 All-trans-lutein	Liu et al., 2004
Chlorophylls	3666 µg/g	 Pheophytin a	Huang et al., 2008
Sterols	NA	 24,24-dimethyl-5α-cholestan-3β-ol	Akihisa et al., 1988

Lignans	325 µg/g root	 Ligballinone 1	Wang et al., 2009
Minerals		Ge (0.56 µg/g), Cu (8.81 µg/g), Zn (24.0 µg/g), Fe (454.7 µg/g) and Mn (78.7 µg/g)	Liang & Sun, 2002

*NA: Not available

1.3 General Health Benefits of Jiaogulan

In East Asia, consumption of jiaogulan can be traced back to the Ming Dynasty (1406 CE) (Blumert et al., 1999). The effects of jiaogulan on health and longevity were recorded in traditional medical books. Recent research into jiaogulan has focused on understanding its role in reducing the risk of chronic diseases such as cardiovascular disease, diabetes, cancer and hepatic disease.

1.3.1 Cardiovascular Disease

Cardiovascular disease is the number one killing disease in the world. The significant cost to treat cardiovascular disease burdens patients, families and nations. Prevention of this disease through diet has therefore been of great interest. Jiaogulan as a folk medicine in East Asia has been promoted for its role in prevention of cardiovascular diseases. In recent publications, several animal studies have been conducted to investigate its cardioprotective effects (Circosta, De Pasquale, & Occhiuto, 2005a; Megalli, Aktan, Davies, & Roufogalis, 2005; Megalli, Davies, & Roufogalis, 2006). A guinea-pig study conducted by Circosta et al. (2005a) compared the protective effect among aqueous extract, isolated gypenosides and verapamil, a Ca-antagonistic drug. This study found that the aqueous extract protected against

coronary spasm, arrhythmias, pressor response, ventricular tachyarrhythmias and lethality similar to verapamil (Circosta et al., 2005a). It also suggested that gypenoside III and gypenoside VIII might be two of the active components of jiaogulan. Another group led by Megalli investigated the hyperlipidemic effects of gypenosides (Megalli et al., 2005). Gypenosides were reported to reduce the plasma triglycerides, total cholesterol and nitrite levels, but not LDL-cholesterol levels of hyperlipidemic rats in both acute (4 days) and chronic (12 days) oral administration. In a subsequent study, gypenosides were able to reduce the triglyceride, total cholesterol and LDL-cholesterol level of Zucker fatty rats (fa/fa). The proposed mechanisms involved in the reduced risk of cardiovascular activities were prevention of calcium ion overload and oxidative stress by reducing the permeability of mitochondria (Circosta et al., 2005a; Schild, Roth, Keilhoff, Gardemann, & Brödemann, 2009); control of nitric oxide levels in macrophages and endothelial cells (Megalli et al., 2006; Tanner, Bu, Steimle, & Myers, 1999); increasing the degradation of triglycerides and reducing very low-density lipoprotein (VLDL) production in the liver by increasing lipoprotein lipase (LPL) activity (Megalli et al., 2005; Megalli et al., 2006), and protection of endothelial cells from oxidant injury (Li & Lau, 1993b). Gypenosides may act as an agonist of Liver X receptor (LXR) and the LXR-gypenoside complex may induce expression of apoE, ABCA1 and LPL. These proteins may decrease the VLDL cholesterol burden and increase the HDL levels (Huang, Razmovski-Naumovski, Salam, Duke, Tran, Duke et al., 2005). The complete mechanism of this effect is not fully understood.

1.3.2 Diabetes

Type 2 diabetes is a major chronic disease that is linked to inherited factors, obesity and aging. The global prevalence of diabetes has attracted attention from the scientific community. Besides the change of life style and drug therapy, herbal products are believed to play a role in prevention and treatment of diabetes. Although jiaogulan has long been consumed as an anti-diabetic beverage, scientific evidence of this effect was not reported until 2004 (Norberg, Hoa, Liepinsh, Van Phan, Thuan, Jörnvall et al., 2004). Jiaogulan extracts or isolated gypenosides were investigated in vitro, and in animal studies and human studies (Adisakwattana, Jiphimai, Prutanopajai, Chanathong, Sapwarabol, & Ariyapitipan, 2010; Hoa, Norberg, Sillard, Van Phan, Thuan, Dzong et al., 2007; Hoa, Phan, Thuan, & Ostenson, 2009; Hung, Hoang, Kim, Jang, Ahn, & Min, 2009; Huyen, Phan, Thang, Hoa, & Ostenson, 2010; Megalli et al., 2006; Nguyen, Gauhar, Hwang, Dao, Park, Kim et al., 2011; Norberg et al., 2004; Xu, Shen, Li, & Hu, 2010; Yassin, 2005; Yeo, Kang, Jeon, Jung, Lee, Song et al., 2008; Zhang, Ji, Chen, Zhou, Luo, Yu et al., 2009). In Megalli and others' (2006) study, after a 5-week treatment, decreased post-prandial glucose levels, and improved glucose tolerance ability were found in the Zucker Fatty rats. The results suggested that insulin receptor sensitivity, but not direct insulin secretion was improved by jiaogulan. This was consistent with a subsequent mice study and a human study (Huyen et al., 2010; Zhang et al., 2009). However, in vitro studies using pancreatic islets found improved secretion of insulin (Hoa et al., 2007; Norberg et al., 2004). Results from another mouse study, conducted by Yeo et al. (2008) supported this conclusion, showing an increase in insulin secretion. The conflicting observation

might be due to the differences in experimental animals, research period (stage of the subjects) and lab protocols. While the exact mechanism is still under investigation, it has been reported to include: increasing the insulin secretion by affecting distal to K-ATP channels and L-type Ca^{2+} channels and suppressing Gi-proteins in cell membrane (Hoa et al., 2007; Norberg et al., 2004); suppressing hepatic glucose output and increase glucose uptake in muscles achieved by stimulation of AMP kinase (Nguyen et al., 2011); delaying the absorption of carbohydrates by inhibiting α -glucosidase and α -amylase's activities (Adisakwattana et al., 2010; Megalli et al., 2006); increasing glycogen storage by stimulating glucokinase's activity (Yeo et al., 2008; Zhang et al., 2009); improving the sensitivity of insulin by inhibiting PTP1B, a known protein to modulate insulin receptor (Hung et al., 2009; Xu et al., 2010; Yassin, 2005); and by suppressing gluconeogenesis (Adisakwattana et al., 2010; Yassin, 2005). These multi-pathway mechanisms indicate potential for jiaogulan as a anti-diabetic herbal product.

1.3.3 Cancer

Due to the genetic factors, and environmental and life habit changes, cancer has become a major threat to human health. Components and extracts of jiaogulan have shown anti-tumor effect in various types of cancer cells and in vivo. In 1998, a mixed jiaogulan and lamp flower extract was found to prevent the carcinogenesis of normal mucosa cells in hamster cheek pouches (Zhou, Wang, Zhou, & Zhang, 1998). Later in 2003, Chiu and colleagues discovered that gypenoside was able to inhibit *N*-acetyltransferase (NAT), an enzyme required for metabolic activation of arylamine carcinogens (Chiu, Chen, & Chung, 2003). Although individual cancer cells might

differ in their growth and proliferation, a series of cancer cell based studies has indicated that gypenoside might induce apoptosis through a similar pathway: gypenosides or extracts might regulate the Bcl-2 family proteins and increase the superoxide dismutase (SOD) level. The reactive oxygen species (ROS) level could be therefore increased, which might lead to the opening of mitochondrial permeability transition pore. As a consequence, cytochrome c could enter the cytosol and induce the expression of caspase 9 and caspase 3, and induce apoptosis (Chen, Lu, Lee, Yeh, & Chung, 2006; Chen, Lu, Tsai, Hsu, Kuo, Yang et al., 2009a; Hsu, Yang, Lu, Yu, Chou, Lin et al., 2011; Lu, Chen, Yang, Chen, Lu, Chiu et al., 2008a; Schild, Chen, Makarov, Kattengell, Heinitz, & Keilhoff, 2010; Wang, Chen, Hsieh, Cheng, & Hsu, 2002). G0/G1 cell cycle arrest in cancer cells has also been observed when treated with gypenosides. The mechanism was believed to be associated with jiaogulan's regulating effects on p53, p27, p16, p15 and cyclin E (Chen et al., 2006; Chen et al., 2009a; Chen, Chung, & Chen, 1999; Hsu et al., 2011; Lu et al., 2008a). Several papers also investigated the inhibitory effect of jiaogulan on invasion and migration (Lu, Chen, Lai, Yang, Weng, Ma et al., 2010; Lu, Tsai, Chen, Hsu, Hsia, Lin et al., 2008b). The signal pathway might involve down-regulation of NF κ B and matrix metalloproteinase-2 & -9 (MMP-2 & -9). Further, a recent study found that gypenoside caused death in human oral cancer cells by causing DNA damage and inhibiting expression of DNA repair genes (Lu et al., 2010). Another mechanism for anti-cancer therapy might be the reduction of multiple drug resistance (MDR) of cancer cells by jiaogulan extract (Huang, Bebawy, Tran, & Roufogalis, 2007c). Many studies indicated that gypenosides might be the major anti-cancer components in

jiaogulan. However, recent studies have reported that flavonoids, carotenoids, chlorophylls and sulfated polysaccharide in jiaogulan also had anti-tumor activities (Chen et al., 2011; Tsai et al., 2010a; Tsai, Wu, & Chen, 2010b). The exact mechanisms of the anti-cancer effects from jiaogulan are still under investigation. But the promising activities imply that jiaogulan might be incorporated into cancer prevention and therapy.

1.3.4 Hepatic and Other Chronic Diseases

Jiaogulan has been used to treat hepatitis in Asia. In 1993, a study found that a water extract of jiaogulan was able to reduce the serum glutamic oxaloacetic transaminase (GOT) & glutamic pyruvic transaminase (GPT) activities in CCl₄-treated rats, which was a direct evidence of liver-protective effect (Lin, Lin, Chiu, Yang, & Lee, 1993). This effect was further investigated in acetaminophen-treated rats. Not only were the serum GOT and GPT activities reduced, but histological improvements were also observed (Lin, Huang, & Lin, 2000). Another 8-week study found that gypenoside was able to prevent the chronic injury and fibrosis of liver (Chen, Tsai, Chen, Chen, & Wang, 2000). Water extract of jiaogulan was also shown to reduce serum uric acid in humans with nonalcoholic liver disease. The antifibrotic effect may involve down-regulation of cyclin D1 and cyclin D3, and inhibition of the signal pathway of PDGF-Akt-p70S6K (Chen, Chen, Wang, Chen, Chang, Hsu et al., 2008). Other mechanisms have yet to be investigated related to the hepatoprotective effects of jiaogulan.

Jiaogulan is also known for its gastrointestinal tract and kidney protective effects (Hesse, Razmovski-Naumovski, Duke, Davies, & Roufogalis, 2007;

Rujjanawate, Kanjanapothi, & Amornlerdpison, 2004). The ability of gypenosides to improve exercise-induced fatigue was reported in mice (Ding, 2010). After 28 days' administration of jiaogulan (50 mg/kg and 100 mg/kg), mice exhibited delayed decrease in blood glucose and prevented increase in lactate. Jiaogulan is reported to have neuroprotective effects against Parkinson's disease and Alzheimer's disease (Choi, Park, Kim, Hwang, Lee, & Lee, 2010; Joh, Yang, & Kim, 2010). The neuroprotective effects may be attributed to jiaogulan's antioxidant effect, including its radical scavenging capacities, increasing antioxidant compounds, enhancing antioxidant enzymes (Wang, Niu, Guo, Gao, Li, Jia et al., 2010; Zhang, Zhao, Gao, Deng, Wang, Xu et al., 2011). Another study has indicated that a gypenoside TN-2 (10-40 mg/kg) was able to ameliorate memory and learning deficits via CREB-BDNF pathway, therefore ameliorate Alzheimer's disease (Winrow, Winyard, Morris, & Blake, 1993). In addition, Lee and others found jiaogulan was an effective agent for skin whitening (Lee, Yoo, Yoo, & Byun, 2007). In the study, ethanol extract of jiaogulan reduced accumulation of extra-cellular melanin of B16 melanoma via inhibition of melanogenesis and melanin transportation (Lee et al., 2007).

1.4 Inflammation, Immunity and Health

Inflammation is a protective and complex process for tissues to remove pathogens, foreign objects and dysfunctional cells. It is initialized by tissue damage, which alters the chemical composition of the interstitial fluid and leads to the process of inflammation. It exhibits symptoms such as redness, swelling, heat and pains. At the cellular levels, there are two major pathways for inflammation: the arachidonic acid/COX pathway and the NF- κ B pathway. In the arachidonic pathway, arachidonic

acid is released from phospholipid when the tissue is injured. This substance is converted into prostaglandins and thromboxanes through the action of cyclooxygenase (COX). Prostaglandins and thromboxanes can induce pain and fever, aggregate platelets, and cause clot formation. The inhibition of COX enzyme activity can reduce the inflammatory symptoms thus COX has become an anti-inflammatory drug target. The other pathway is related to NF- κ B, a protein complex that controls the transcription of a large number of inflammatory proteins. In its normal state, NF- κ B, binds to I κ B proteins and is inactive. Stimuli including trauma, viral infections, ultraviolet radiation, free radicals, and the cytokines such as TNF α and IL-1 β can degrade I κ B, and therefore activates NF- κ B. NF- κ B translocates to the nucleus and binds to target genes to activate inflammatory gene expression. Acute inflammation protects the body from infection, while an improperly regulated immune system could lead to prolonged inflammation, and thus cause a host of diseases. Chronic inflammation has been linked with tumor development, allergies and autoimmune diseases (Grivennikov, Greten, & Karin, 2010).

Recently, jiaogulan's anti-inflammatory components and effects have been investigated. A water extract of jiaogulan showed anti-inflammatory effect by inhibiting paw edema rate in rats (Lin et al., 1993). Several later studies indicated that extracts or gypenosides of jiaogulan can effectively alleviate asthma by decreasing bronchial resistance (PVP), attenuating airway hyperresponsiveness (AHR), and inhibiting eosinophil infiltration (Circosta, De Pasquale, Palumbo, & Occhiuto, 2005b; Huang, Tran, Roufogalis, & Li, 2007a; Huang, Tran, Roufogalis, & Li, 2007b; Huang, Kuo, Li, Yang, Liou, & Shen, 2008b; Hung, Thu, Cuong, Hung, Kwack, Huh

et al., 2010; Liou, Huang, Kuo, Yang, & Shen, 2010). T and B lymphocytes from spleen are associated with AHR and eosinophil infiltration in asthma. Cytokines and antibodies secreted from the T helper (Th) cells are the main mediators in asthma. Jiaogulan has been reported to decrease secretion of IL-4, IL-5, IL-13, IgE and IgG1, and increase expression of IFN- γ in Th2 cells. It is also able to promote Th1 cytokine expression in spleen cells (Huang, 2007; Liou et al., 2010). Jiaogulan might also alleviate asthma by suppressing eotaxin, a C-C chemokine that recruits eosinophils in human bronchial epithelial cells (Hung et al., 2010). The anti-inflammatory mechanism has been partially unveiled. Aktan et al. discovered that gypenoside was able to suppress NO synthesis by directly inhibiting the catalytic activity of iNOS enzyme and LPS-induced NF κ B activity (Aktan, Henness, Roufogalis, & Ammit, 2003). Huang et al. indicated that NF κ B was inhibited via a PPAR α dependent pathway by jiaogulan. And in THP-1 monocytic cells, tissue factor (TF) is downregulated while in HUVEC, VCAM-1 is downregulated, both via the PPAR α dependent pathway (Huang, Li, Razmovski-Naumovski, Tran, Li, Duke et al., 2006; Huang et al., 2007a; Huang et al., 2007b). Further research is needed to better understand jiaogulan's anti-inflammatory mechanism.

Jiaogulan has also been investigated for its immunoregulatory effect. As an adjuvant, gypenosides increased OVA-, LPS-, and ConA-stimulated splenocyte proliferation, and also increase OVA-specific antibodies, with slight haemolytic activities (Sun & Zheng, 2005). Extracts of jiaogulan were able to enhance the production of antibodies such as IgM, IgG2a, IgA, and IgG1 in serum and spleen

cells of mice. Cytokines were increased in Con A-stimulated splenocytes of jiaogulan-treated mice (Huang, Kuo, Li, Yang, Liou, & Shen, 2007d).

1.5 Reactive Species, Oxidative Stress and Health

Reactive species include both reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Adisakwattana et al., 2010). The most prevalent ROS include peroxy radicals (ROO^\bullet), superoxide anion radical ($\text{O}_2^{\bullet-}$), singlet oxygen ($^1\text{O}_2$), hydroxyl radical ($^\bullet\text{OH}$) and hydrogen peroxide (H_2O_2). The superoxide anion radical ($\text{O}_2^{\bullet-}$) seems to play a central role as major oxidative reactions start with this radical (Diplock, Charleux, Crozier-Willi, Kok, Rice-Evans, Roberfroid et al., 1998). The hydroxyl radical ($^\bullet\text{OH}$) is the most reactive species with an estimated half-life of about 10^{-9} s, thus initiates immediate reaction where it is generated (Diplock et al., 1998). RNS generally include peroxynitrite ($^\bullet\text{ONOO}$), nitric oxide ($^\bullet\text{NO}$) and nitrogen dioxide ($^\bullet\text{NO}_2$). Nitric oxide radical ($^\bullet\text{NO}$) is a signaling compound in endothelial cells. The production and release of nitric oxide from endothelial cells relaxes smooth muscle cells and thus reduces blood pressure. Excessive nitric oxide, however, might combine with superoxide anion radical to induce lipid peroxidation (Diplock et al., 1998). Notably, the term free radical normally refers to unpaired electrons in a molecule. But reactive species discussed here also include highly reactive molecules, such as singlet oxygen and peroxide, which would potentially damage the macromolecules. ROS and RNS can be generated endogenously. A good example would be the radicals produced by immune macrophage cells to eliminate foreign invasion. ROS/RNS may be generated and serve as signaling compounds such as

nitric oxide stated above. Reactive species can also come from external sources. The dietary intake of certain types of food may introduce prooxidants such as quinones. In addition, smokers are believed to induce a significant higher level of free radicals. And pollutants such as ozone and nitrogen dioxide might affect the reactive species level (Kelly, 2003).

Oxidative status *in vivo* is mediated through an oxidative control system. It includes enzymes and non-enzymatic compounds. The major enzymes involved in the control of reactive species are: superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and glutathione reductase (GSH reductase). The superoxide dismutase (SOD) catalyzes the dismutation of reactive $O_2^{\bullet-}$ into oxygen and hydrogen peroxide. Glutathione peroxidase (GPx) and catalase are responsible for converting the hydrogen peroxide into water. And glutathione reductase (GSH reductase) is able to regenerate GSH, an endogenous antioxidant from its oxidized form GSSG. Non-enzymatic compounds, including GSH, Vitamin C, Vitamin E, Co-enzyme Q10, as well as metal ions such as Fe, Cu or Se ions are also affecting the oxidative status (Diplock et al., 1998). These factors are tightly regulated in normal organisms.

Oxidative stress refers to an imbalance between the production of reactive species and the antioxidant defense *in vivo*. It could result from low levels of antioxidants or overproduction of reactive species (Halliwell & Whiteman, 2004). Reactive species are responsible for the oxidative damage of biological macromolecules such as DNA, lipids and proteins. Excessive amount of reactive species may be involved in pathogenic processes of numerous of diseases such as

cardiovascular disease, cancer, cataract, rheumatoid arthritis and nervous diseases (Diplock et al., 1998).

1.6 Antioxidant and Health

The term “antioxidant” has several meanings. To food engineers, it might indicate reagents that can preserve the quality of foods from oxidation; to chemists, it means compounds or foods that can scavenge free radicals; to nutritionists or biochemists, it might also mean compounds or foods that can up-regulate the antioxidant defense system in vivo (Finley, Kong, Hintze, Jeffery, Ji, & Lei, 2011). Only the latter two are included in the scope of this research.

Antioxidants work through different mechanisms alone or combined. One of the major ways we have seen and tested by chemical methods is direct radical quenching and metal chelating capacities. Antioxidants may also be involved in gene and protein expression of those antioxidant enzymes, sequestration of pro-oxidants, modification of ratio of reducing substances, effect on signaling pathways.

Many phytochemicals are believed to have antioxidant effects. Flavonoids have been demonstrated to have efficient radical scavenging properties. The relationship between radical scavenging properties and structure is well understood and documented (Rice-Evans, Miller, & Paganga, 1996). Recent studies have also indicated that flavonoids are able to regulate antioxidant enzyme expression and affect cell signaling pathway in many processes (Stevenson & Hurst, 2007). It's noteworthy that flavonoids are poorly absorbed due to their structures. Less than 5% of flavonoids are present in blood, indicating that the indirect biological effect, rather

than the direct scavenging effect might be playing the major health beneficial role. Saponins have also been reported to have antioxidant effect (Chen, 2011; Huong, Matsumoto, Kasai, Yamasaki, & Watanabe, 1998; Li et al., 1993b; Liu, Luo, Liu, Chen, Wang, & Sun, 2003). However, like many other compounds, saponins may act in dual roles — the high concentration and certain types of saponin may lead to pro-oxidant effect (Liu et al., 2003).

Diets with high natural antioxidants, including vitamin C, β -carotene, α -tocopherol, and polyphenols have been linked with the reduced risk of chronic diseases according to epidemiological studies and human intervention studies (Diplock et al., 1998). However, several studies with high β -carotene intake showed negative or no effect on cancer or cardiovascular diseases (Hennekens, Buring, Manson, Stampfer, Rosner, Cook et al., 1996; Omenn, Goodman, Thornquist, Balmes, Cullen, Glass et al., 1996). Similar cases have been reported with vitamin E (Christen, Gaziano, & Hennekens, 2000). These studies indicate that under certain conditions of the organism, these compounds may act as pro-oxidants. It is also notable that a study conducted in 2009 found antioxidant treatment (vitamin C & E) counteracted the positive effect of exercise on amelioration of insulin resistance and promotion of endogenous antioxidant defense capacity (Ristow, Zarse, Oberbach, Klöting, Birringer, Kiehnkopf et al., 2009). It has been suggested that a certain level of ROS may be important to trigger antioxidant response, and that a high dose of antioxidant, resulting in removal of too many ROS, may be detrimental to human health (Finley et al., 2011).

Jiaogulan is a botanical rich in antioxidants. Early in 1993, Li and his colleague found that gypenosides decreased radicals in phagocytes, and inhibited the lipid peroxidation in liver microsomes and vascular endothelial cells (Li et al., 1993b). It was able to reverse the decreased membrane fluidity of liver microsomes and mitochondria, increase the mitochondrial enzyme activity in vascular endothelial cells and decrease intracellular lactate dehydrogenase (LDH) activity. Later, a series of studies indicated that jiaogulan can interfere with other cellular enzymes such as SOD and xanthine oxidase. In addition, jiaogulan extracts were reported to scavenge superoxide radicals (Lin et al., 2000). Taken together, these studies indicate that jiaogulan may regulate the reactive status by radical scavenging activity and modification of antioxidant enzymes.

1.7 Diploid and Tetraploid

Genotype is considered an most important factors in determining the chemical compositions in botanicals. Polyploidization, a common method for genetic modification, may be an effective way to alter the chemical properties and may yield a desirable plant genotype. For example, wheat with different chromosome ploidy performed differently in drought tolerance and yield performance (Xiong, Li, & Zhang, 2006). It has been reported that tetraploid ginger had much higher total gingerol content than its diploid counterpart (Sanwal, Rai, Singh, & Buragohain, 2010). While the majority of jiaogulan in nature is in diploid form, most of the studies did not disclose the genotypes of the botanical. It is unknown how genotype affects the nutraceutical and health properties for jiaogulan.

1.8 HPLC/MS Fingerprinting

High-performance liquid chromatography (HPLC) is a technique for physical separation of compounds in solution. Generally HPLC consists of a reservoir of mobile phase, a degasser, a pump, an injector (sampler), a separation column, and detector(s). Different compounds have different partitioning behavior in mobile phase and stationary phase, thus have different retention times on the column. There are many types of HPLC procedures according to the stationary and mobile phase combination. The most widely applied is reverse phase HPLC (RP-HPLC), which has a non-polar stationary phase and a moderately polar mobile phase. The common stationary phase is silica-based octadecylsilane (ODS), a.k.a. C18. The choice of detectors varies with the compounds to be detected. The most common detectors include UV/Vis detector, fluorescence detector, refractive index detector, and mass spectrometry. The UV/Vis detector is most prevalent because of its low cost and universal use. But its relatively low sensitivity and selectivity makes detection and characterization of certain compounds difficult.

With the improvement of technology, mass spectrometry (MS) coupled with HPLC or gas chromatography (GC) has become a popular method for detection of protein/peptides, oligosaccharides, vitamins, drug and metabolites, and phytochemicals due to its high sensitivity, selectivity, and ability to obtain ion fragments and structure information. MS works by ionizing chemical compounds to generate charged molecules or fragments. And their mass-to-charge ratios are measured in the detector. MS usually consists of three parts: an ion source, which converts molecules into ions; a mass analyzer, in which ions are separated according

to their mass-to-charge ratios; a detector, which measures and determines the intensity of the ion signals.

Quality control is an important step in manufacturing. The quality and flavor of food products or nutraceuticals, especially plant-based materials, can be greatly influenced by different varieties, parts of plant, cultivation areas, climatic conditions and post-harvest processing (Moore et al., 2006a; Whent et al., 2009; Yang, An, Jiang, Tang, Gao, Zhao et al., 2011). Adulteration of raw materials is also a concern worldwide on the safety and quality of foods and medicines (Moore, Spink, & Lipp, 2012). A detection of single or several chemicals is not sufficient since the quality of food and botanical relies on a number of components. A more reliable and effective quality control would need to identify multiple active ingredients. Chemical fingerprinting methods, which compare the chemical profiles of multiple ingredients simultaneously, is endorsed by the World Health Organization (WHO) as an effective strategy for quality assessment.

HPLC alone, or with the help of MS, could generate chromatograms of samples, which consists of “fingerprint” peaks representing characteristic chemical components. Using this approach, an authentic sample of a certain material is selected as a reference fingerprint (RF). The most obvious peak of the RF is selected as the reference peak (RP). The chromatogram is first normalized according to the area of the RP. Retention time alignment of peaks in these chromatograms is also used. Aside from the visual discrimination of the chromatograms of samples, chemometric data analyses such as principal component analysis (PCA) can also be utilized to assess the similarity of chromatographic fingerprints. Although jiaogulan is a well-known

botanical and commercial jiaogulan products have been on the market for some time, little work has been done on development of a chromatography fingerprinting method for quality control.

1.9 Regulation and Marketing

Currently jiaogulan is produced outside of the United States. For importing jiaogulan as food products, the importers need to ensure the products are “safe, sanitary, and labeled according to U.S. requirements”. To be added to foods as an ingredient in the US, jiaogulan must conform the regulation as Generally Recognized As Safe (GRAS) or as food additives. As food additives require lengthy evaluation by Food and Drug Administration (FDA), GRAS status is a more commonly used approach (Joy, 2006). According to sections 201(s) and 409 of the Federal Food Drug and Cosmetic Act, “any substance that is intentionally added to food is a food additive, that is subject to premarket review and approval by FDA, unless the substance is generally recognized, among qualified experts, as having been adequately shown to be safe under the conditions of its intended use, or unless the use of the substance is otherwise excluded from the definition of a food additive.” One does not need FDA’s approval to sell or market substances that are considered GRAS in the US. Companies may initiate a private expert panel to determine jiaogulan as GRAS for its intended purpose based on scientific procedure and jiaogulan’s long consumption history by a large population in Asia. A notification of GRAS can also be submitted to FDA voluntarily, and if the agency doesn’t disagree with the submitters’ conclusions, published in FDA’s GRAS inventory list, and may promote the marketing of jiaogulan (Joy, 2006). To date, no GRAS notification on jiaogulan

for food incorporation has been filed yet, and it is unknown whether a private company has carried out a set GRAS determination.

The dietary supplement in the US has a similar regulation. Jiaogulan, as a dietary ingredient, might be incorporated into dietary supplement. A new dietary ingredient is defined by statute as “a dietary ingredient that was not marketed in the United States before October 15, 1994.” Whether jiaogulan is a new dietary ingredient is worth discussing. If it is considered an existing dietary ingredient, as some of the manufacturers claimed, it can be added into dietary supplement without notification of FDA. On the other hand, jiaogulan might be treated as a new dietary ingredient. In this case, notifications for jiaogulan should be submitted to the FDA which must contain level of the new dietary ingredient, conditions of use of the product, the history of use, and other evidence of safety. The agency will then review the information for adequacy and safety concerns. The dietary supplement manufacturing must also meet the current Good Manufacturing Practices (cGMP) focusing on “practices that ensure the identity, purity, quality, strength and composition of dietary supplements” (FDA, 2009).

Jiaogulan has been largely imported into the US markets as tea and supplements. As stated above, jiaogulan has been shown to contain various phytochemicals and have numerous biological effects and is easy and not expensive to grow. Jiaogulan has a relatively small market share, mostly due to its unpopularity in the US. As more and more studies are published, the potential need may grow.

In summary, jiaogulan has shown potential beneficial health properties, and contains abundant amounts of bioactive components. However, details of the health

promoting mechanism have not been fully elucidated. Jiaogulan from different sources, different genotypes, and different parts of the plant may vary in chemical compositions and health beneficial properties but current research has not studied those factors. Therefore, the specific objectives of this dissertation research are:

1. To assess and compare different jiaogulan samples for their phytochemical compositions, and antioxidant, anti-proliferative and anti-inflammatory properties
2. To assess and compare jiaogulan of different genotypes and derived from different plant parts for their phytochemical compositions, and antioxidant, anti-proliferative and anti-inflammatory properties
3. To develop a chromatographic fingerprinting method for jiaogulan of different genotypes and plant parts using LC/MS

Chapter 2: Chemical Composition of Five Commercial

Gynostemma pentaphyllum Samples and Their Radical

Scavenging, Anti-proliferative, and Anti-inflammatory

Properties

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2.1 Abstract

Five *Gynostemma pentaphyllum* samples were investigated and compared for their chemical compositions, and their antioxidant, anti-proliferative and anti-inflammatory effects. Extracts (50% acetone, 75% ethanol, and 100% ethanol) of the five GP samples (GP1-5) differed in their total phenolic, saponin, and flavonoid contents, and in their rutin and quercetin concentrations. The highest level of total flavonoids was 63.5 mg rutin equiv/g in GP4, and the greatest total phenolic content was 44.3 mg gallic acid equiv/g in GP1 with 50% acetone as the extraction solvent. GP2 had the highest total saponin content of 132.6 mg /g with 100% ethanol as the extraction solvent. These extracts also differed in their scavenging capacity against DPPH and hydroxyl radicals, although they all showed significant radical scavenging capacity. The 100% ethanol extracts also showed dose-dependently strong inhibition on IL-6 and Ptg2 mRNA expression and weak inhibition on TNF- α mRNA expression. In addition, GP1 had highest anti-proliferative activity at 3.2 mg equiv/mL concentration in HT-29 human colon cancer cells. The results from this

study will be used to promote the application of *G. pentaphyllum* for improving human health.

2.2 Introduction

Gynostemma pentaphyllum Makino is a perennial liana plant, which grows in Asia including China, Japan, Korea, Thailand, and Malaysia. Its aerial part has been traditionally used in food, tea, and folk medicines (Wang et al., 2007). Growing evidence has indicated that *Gynostemma pentaphyllum* may play a role in reducing the risk of cardiovascular diseases (Tanner et al., 1999), and may have hypoglycemic (Yeo et al., 2008), anti-inflammatory (Lin et al., 1993), anticancer (Chen et al., 2009a; Lu et al., 2008b) and hepatoprotective activities (Chen, Wang, Chen, Shee, Chen, Chen et al., 2009b) with little concern of toxic effects (Attawish, Chivapat, Phadungpat, Bansiddhi, Techadamrongsin, Mitrijit et al., 2004). Flavonoids and gypenosides, the saponins of *Gynostemma pentaphyllum*, are considered the primary phytochemicals contributing to the health properties (Wang et al., 2007). In 1993, Li and Lau reported that gypenosides, suppressed lipid peroxidation and cell injury induced by hydrogen peroxide (Li et al., 1993b). Later in 1993, *Gynostemma pentaphyllum* gypenosides were shown to reduce superoxide anion and hydrogen peroxide contents in human neutrophils and diminish chemiluminescent oxidative burst triggered by zymosan in human monocytes and murine macrophages (Li et al., 1993b). The gypenosides also suppressed lipid peroxidation in liver microsomes and vascular endothelial cells under the experimental conditions. These antioxidant activities were considered important for the beneficial effects of *Gynostemma pentaphyllum* in preventing aging-associated health problems including chronic

inflammation and cardiovascular diseases (Li, Jiao, & Lau, 1993a; Li et al., 1993b). In addition, one of the *Gynostemma pentaphyllum* polysaccharides showed scavenging activity against superoxide radicals and inhibitory effects on self-oxidation of 1,2,2-phentriol, suggesting its potential as an antioxidant (Wang et al., 2007). The possible chemical and biochemical mechanisms involved in their antioxidative actions may include but are not limited to termination of free radical mediated oxidative chain reaction, induction of antioxidant enzymes, and reduction of peroxides. To date, the radical scavenging properties of the non-sugar *Gynostemma pentaphyllum* components have not been investigated.

It is well recognized that the phytochemical composition and health properties of botanical materials may vary greatly because of the genotype, growing environment, and the interactions between genotype and environment (Moore et al., 2006a; Whent et al., 2009). Many *Gynostemma pentaphyllum* products are commercially available and used for improving human health. It is critical for optimal health benefits of these *Gynostemma pentaphyllum* products to clearly understand if there is any difference among these materials and how different they might be. No study has investigated the variations in the chemical composition and health properties of these commercial *Gynostemma pentaphyllum* materials grown from different locations in Asian countries.

In the present study, five commercial *G. pentaphyllum* samples including three samples grown in selenium-rich soil were extracted with 50% acetone, 75% ethanol and 100% ethanol, and examined for their total phenolic, total saponin, total flavonoid, rutin and quercetin contents, and their scavenging capacities against DPPH

and hydroxyl radicals. The 100% ethanol extracts were also examined and compared for anti-proliferative activity in HT-29 human colon cancer cells, and potential anti-inflammatory effects. The anti-inflammatory effects were determined as their capacities to suppress TNF- α , IL-6 and COX-2 gene expression in cultured mouse J774A.1 macrophages. The results from this study will be used to promote the better use of *Gynostemma pentaphyllum* for improving human health.

2.3 Materials and Methods

2.3.1 Materials

Three *Gynostemma pentaphyllum* Makino samples were obtained from Asian Citrus Holdings Limited (Hong Kong) and other two samples were purchased from grocery markets in the United States. The five *Gynostemma pentaphyllum* samples were coded as GP1, GP2, GP3, GP4 and GP5. Iron (III) chloride, fluorescein, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), and 2,2-diphenyl-1-picrylhydrazyl radical (DPPH[•]) were obtained from Sigma-Aldrich (St. Louis, MO). 2,2'-azinobis (2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals USA (Richmond, VA). Thirty percent ACS-grade H₂O₂ was purchased from Fisher Scientific (Fair Lawn, NJ). The mouse J774A.1 macrophage cell line was purchased from ATCC (Manassas, VA). Lipopolysaccharide (LPS) was purchased from Sigma-Aldrich (St. Louis, MO). Ultrapure water was prepared by an ELGA Purelab ultra Genetic polishing system with < 5 ppb TOC and resistivity of 18.2 m Ω (Lowell, MA) and was used for all experiments. The cell culture media were

purchased from Invitrogen (Carlsbad, CA). All other chemicals and solvents were of the highest commercial grade and used without further purification.

2.3.2 Sample Preparation

Botanical samples were ground to a particle size of 40-mesh using a household coffee grinder. For ethanol extraction, approximately twelve grams of ground samples were extracted in 250 mL pure ethanol for 5 h using a Soxhlet apparatus. For 50% acetone and 75% ethanol extractions, approximately two grams of ground samples were extracted in 20 mL of the solvent at ambient temperature and then filtered using 45-micron syringe filter (Fisher Brand, Pittsburg, PA). Extracts were kept in dark until testing.

2.3.3 Determination of Water Content and Se Concentration

The ground samples were weighed before and after being placed in an oven at 100 °C to constant weight. The weight change was used to calculate the water content of the sample. Se concentration was determined by inductively coupled plasma dynamic reaction cell mass spectrometry (ICP-DRC-MS) in a commercial analytical lab (Applied Speciation and Consulting LLC, Bothell, WA).

2.3.4 Total Fiber Content

Total fiber contents were measured with a commercial kit purchased from Megazyme International Ireland Ltd. (Wicklow, Ireland) according to the previously reported procedure (Yu & Perret, 2003).

2.3.5 Total Phenolic Content (TPC)

TPC of antioxidant extraction was measured according to a laboratory procedure described previously (Yu, Haley, Perret, & Harris, 2002). Briefly, 100 μ L of extract was mixed with 500 μ L of the Folin-Ciocalteu reagent, 1.5 mL of 20% sodium carbonate, and 1.5 mL ultrapure water. Gallic acid was used as the standard. Absorbance was read at 765 nm on a Genesys 20 spectrophotometer (Thermo Scientific, Waltham, MA) after 2 hours of reaction at ambient temperature in dark. Reactions were conducted in triplicate and results were reported as mg gallic acid equivalents (GAE) per gram of the botanical sample.

2.3.6 Total Saponin Content (TSC)

The total saponin content was determined using the method described previously (Hiai et al., 1976). Briefly, the extract (100 μ L) was mixed with 200 μ L 5% vanillin in acetic acid and 800 μ L 70% perchloric acid. The mixture was incubated at 60 $^{\circ}$ C for 15 min. A commercial *Gynostemma pentaphyllum* gypenoside extract was used as the quantification standard. The absorbance was read at 550 nm on a Genesys 20 spectrophotometer (Thermo Scientific, Waltham, MA) after sample cooled down to ambient temperature.

2.3.7 Total Flavonoid Content (TFC)

Total flavonoid content was determined by the aluminum colorimetric method described previously (Quettier-Deleu, Gressier, Vasseur, Dine, Brunet, Luyckx et al., 2000). Briefly, the sample extract was mixed with 1 mL 5% sodium nitrite. After 6 min, 10% of aluminum nitrate was added. Then 4% sodium hydroxide was added into

the mixture. Rutin was used as the standard. Absorbance was read at 502 nm on a Genesys 20 spectrophotometer (Thermo Scientific, Waltham, MA) after 15 min of reaction. The results were reported as mg rutin equivalents per gram of botanicals.

2.3.8 Flavonoid Profile

The flavonoid profile was determined by HPLC-DAD analysis according to the protocol described previously with minor modification (Chen et al., 2007). The extracts were re-dissolved in methanol and filtered through 0.45 μ m membrane filters. Compounds were separated on a phenomenex Synergi 4u hydro-RP 80A column (250 mm \times 2.0 mm, 4 μ m). The HPLC separation was accomplished using a two-solvent gradient system. Briefly, mobile phase A consisted of 0.1% formic acid (FA) in H₂O and mobile phase B consisted of 0.1% FA in acetonitrile. The initial ratio of A to B was 80:20 (v/v); this was changed linearly to 35:65 in 25 min, to 5:95 after 26 min, and then reset to 80:20 from 27 min to 32 min. The wavelength for UV detection was 370 nm. The flavonoids were identified via comparison to standards for retention time and UV spectra, and were quantified via the area under the curve using external standards.

2.3.9 Relative DPPH Radical Scavenging Capacity (RDSC)

The RDSC values were determined following a procedure described previously (Cheng, Moore, & Yu, 2006). Briefly, 100 μ L of sample extract, solvent, or standard solution of trolox were added to 100 μ L of freshly prepared DPPH[•] solution to initiate antioxidant-radical reaction. The absorbance of the reaction mixture was measured at 515 nm during 40 min of reaction. An initial DPPH[•]

concentration of 100 μM was used for all reactions. RDSC values were calculated using areas under the curve relative to trolox standards. Results are expressed as micromoles of trolox equivalents (TE) per gram of botanical sample.

2.3.10 ESR Spin-Trapping Assay for DPPH Radical Scavenging Capacity

The ESR measurements were conducted according to a previously described procedure (Cheng, Zhou, Yin, & Yu, 2007). Final concentration of sample extracts in the reaction mixture was 4 mg and 8 mg botanical equivalents per mL of DMSO. Botanical equivalent represents the original plant material equivalent. The final concentration of DPPH radical was 0.4 mM. The ESR spectra were recorded at 5 and 15 min of each reaction at ambient temperature with microwave power of 2 mW, field modulation frequency of 100 kHz, and modulation amplitude of 3.79 G.

2.3.11 Hydroxyl Radical Scavenging Capacity (HOSC)

HOSC assay was conducted according to a previously published laboratory protocol (Moore, Yin, & Yu, 2006b) using a Victor³ multilabel plate reader (PerkinElmer, Turku, Finland). Standard solutions of trolox were prepared in 50% acetone. Reaction mixtures consisted of 170 μL of 9.28×10^{-8} M FL, 30 μL of standard, sample extract, or blank, 40 μL of 0.1990 M H_2O_2 , and 60 μL of 3.43 mM FeCl_3 . Fluorescence was measured every minute for 3 h with an excitation wavelength of 485 nm and an emission wavelength of 535 nm. HOSC values are expressed as micromoles of trolox equivalents (TE) per gram of sample.

2.3.12 HT-29 Colon Cancer Cell Proliferation Inhibition

100% ethanol extracts were used for anti-proliferative effect analysis. After removing the solvent, the antioxidant extracts were re-dissolved in DMSO and the final concentrations of *G. pentaphyllum* in cell media were 0, 0.032, 0.32, 3.2 mg botanical equivalents per mL. HT-29 human colorectal adenocarcinoma cell proliferation inhibition was investigated according to a previously reported protocol (Slavin, Kenworthy, & Yu, 2009). The cells were grown at 37 °C and 5% carbon dioxide in the McCoy's 5A media supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic. The treatment media contained *G. pentaphyllum* extracts at concentrations of 0, 0.032, 0.32, 3.2 mg botanical equivalents/mL, with 0.3% DMSO. ATP-Lite 1 step kit (Perkin Elmer Life and Analytical Sciences, Shelton, CT) was used to determine cell proliferation. The emitted luminescence was determined using a Victor³ multi-well plate reader (Perkin Elmer, Turku, Finland) immediately prior to treatment and at 4, 24, 48, 72 and 96 h after initial treatment. Treatment media were replaced every 24 h until a reading was taken on that plate.

2.3.13 TNF- α , IL-6 and COX-2 Gene Expression Inhibition in Mouse J774A.1

Macrophage Cells

The DMSO solution was used for anti-inflammatory study, and the final concentrations of *G. pentaphyllum* in cell media were 0, 0.0256, 0.128, 0.64, 3.2 mg botanical equivalents per mL. Mouse J774A.1 macrophages were cultured in 12-well plates overnight and reached the confluence of 80%. The cells were first treated with cell media containing *G. pentaphyllum* extracts for 2 h. Then lipopolysaccharide

(LPS) was added at an initial concentration of 0.5 µg/mL. Cells were incubated at 37 °C and 5% CO₂ for 24 h.

The cells were used for RNA isolation and Real-time PCR. RNA isolation and Real-time PCR of TNF- α , IL-6 and Ptgs2 was performed following the previous reported protocol (Trasino, Kim, & Wang, 2009; Zhou, Lutterodt, Cheng, & Yu, 2009). After 24 h incubation, the TRIzol reagent (Invitrogen) was used for total RNA isolation, and StrataScript First Strand complementary DNA Synthesis kit (Stratagene) was used to reverse transcribe complementary DNA. Real-time PCR was performed on ABI Prism 7000 Sequence Detection System (Applied Biosystems) using TaqMan Universal PCR Master Mix. The TaqMan Assay-On-Demand was purchased from Applied Biosystems: Il6 (Mm00446190), Tnf (Mm00443258), Ptgs2 (Mm01307329). The mRNA contents were normalized to an internal control, Tbp (Mm00446973) mRNA. The following amplification parameters were used for PCR: 50 °C for 2 min, 95 °C for 10 min, and 46 cycles of amplification at 95 °C for 15 s and 60 °C for 1 min.

2.3.14 Statistic Analysis

Data were reported as mean \pm SD for triplicate determinations. One-way ANOVA and Tukey's test were employed to identify differences in means. Statistics were analyzed using SPSS for Windows (version rel. 10.0.5, 1999, SPSS Inc., Chicago, IL). Correlation was analyzed using a two-tailed Pearson's correlation test. Statistical significance was declared at $P < 0.05$.

2.4 Results and Discussion

2.4.1 Total Phenolic Content

The total phenolic contents are estimated using three solvent systems including 50% acetone, 75% ethanol, and 100% ethanol (**Table 2.1**). The highest TPC value of 44.3 mg gallic acid equivalents (GAE)/g *Gynostemma pentaphyllum* botanical was detected in the GP1 with 50% acetone as the extraction solvent, which was followed by that of 43.2 mg GAE/g in the GP4 sample using the same solvent. The TPC values ranged from 12.3 to 44.3 mg GAE/g for 50% acetone extracts, 10.2-37.5 mg GAE/g for 75% ethanol extracts, and 6.7-33.6 mg GAE/g for 100% ethanol extracts (**Table 2.1**). The total phenolic content of GP1-5 (6.7-44.3 mg GAE/g) was high comparable to that in the defatted black raspberry seed flour (41.2 mg GAE/g flour) and the defatted red raspberry seed flour (25.1 mg GAE/g flour) (Parry, Su, Moore, Cheng, Luther, Rao et al., 2006). Phenolic compounds are considered the primary contributors for the overall antioxidant activity in botanical samples (Havsteen, 2002).

Also noted was that the TPC values varied significantly between the botanical samples regardless of extraction solvent. The extraction solvent might significantly affect the TPC estimation. Together, these data suggested the structural diversity of the phenolic compounds in the GP1-5 samples. In addition, 50% acetone extract had the highest TPC value compared to the corresponding 100% and 75% ethanol extracts for each *G. pentaphyllum* botanical samples, indicating that 50% acetone is a better solvent for extracting total phenolics from *G. pentaphyllum* botanicals.

2.4.2 Total Saponin Content

Saponins are the most notable compounds in *G. pentaphyllum*. *G. pentaphyllum* is one of the few plants that have ginsenosides once considered unique in Ginseng. Several studies have showed the possible association between saponin content and the potential anti-cancer (Liu, Huang, Cui, Huang, Mao, Ji et al., 2009) and anti-inflammatory effects of *G. pentaphyllum* (Park & Cho, 2009). In the present study, total saponin contents were determined in the five GP samples to reflect their potential health properties. GP2 had the greatest total saponin content of 132.6 mg/g botanical using a commercial total GP saponin extract as the standard. The five GP samples differed significantly in their total saponin contents with the same extraction solvent (**Table 2.1**). For each GP sample, the 100% ethanol extract had the highest total saponin content with a range of 65-133 mg/g sample, and the 50% acetone extract had the lowest values ranging from 23.6 to 90.2 mg/g (**Table 2.1**), suggesting 100% ethanol is a better solvent for saponin quantification for GP samples. Interestingly, GP5 had a second highest total saponin content value using 100% ethanol for extraction, but the lowest saponin content within 50% acetone as the extraction solvent. GP sample with greatest total saponin content may not have highest TPC value. Taking together, these data indicate the different saponin compounds may be presented at different ratios in the individual GP samples.

2.4.3 Total Flavonoid Content, and Rutin and Quercetin Concentrations

Flavonoids are a group of compounds abundant in citrus, tea, and wine. They have been considered to have antioxidative and anti-proliferative effects (Iwashita,

Kobori, Yamaki, & Tsushida, 2000; Ren, Qiao, Wang, Zhu, & Zhang, 2003). In the present study, total flavonoid content was examined using a colorimetric method and individual flavonoids compounds were identified and quantified by HPLC method. As shown in **Table 2.1**, GP4 showed the highest total flavonoids content among the five GP samples regardless of extraction solvent. The greatest total flavonoid value of 64 mg rutin equivalents (RE)/g botanical was obtained for GP4 using 50% acetone in extraction, and followed by that of 54 and 36 mg RE for 50% acetone and 75% EtOH extraction solvent, respectively. This range (36-64 mg RE/g) was significantly higher than total flavonoids content in GP1, GP2, GP3, and GP5 samples with any of the three extraction solvents. None of the three solvents was necessarily better than the other two for total flavonoids estimation in GP samples, suggesting that these GP samples may differ greatly in their flavonoid compound profile and composition.

Rutin and quercetin were the primary flavonoids in *G. pentaphyllum* samples according to previous reports (Kao et al., 2008). Rutin and quercetin were further examined in each of the extracts using a HPLC method. GP1-4 had significant amount of rutin content whereas there was no rutin detectable in GP5 using all three solvents in extraction. Among all samples, GP1 showed the highest rutin content (11235 µg/g) with pure ethanol as the extraction solvent. GP3 had the second highest rutin content for pure ethanol extract, the highest value for 50% acetone and 75% ethanol extracts. GP4 had the lowest rutin content (579-1409 µg/g) among GP1-GP4 regardless of extraction solvent. None of the three solvents was necessarily superior to the other two for rutin extraction. The quercetin content in GP1 (4907-7432 µg/g) was at least 10 times higher than that in GP2-4 (118-549 µg/g) for all solvent extracts.

75% ethanol extract of GP1 showed the greatest quercetin content among all the samples using different solvents (7432 $\mu\text{g/g}$). Since the aglycone moiety of rutin is quercetin, and rutin could be converted to quercetin *in vivo*, the rutin content was converted into quercetin equivalent for discussion (**Table 2.1**). The total quercetin equivalent ranged from 1.4 to 42.5 $\mu\text{mol/g}$ sample, except GP5 with no detectable rutin or quercetin. GP1 had a much higher value (21.2, 37.6 and 42.5 $\mu\text{mol QE}$ (quercetin equivalent)/g for 50% acetone, 75% EtOH and 100% EtOH extract respectively) than other samples. GP4 had the lowest value (1.4-3.1 $\mu\text{mol/g}$) among GP1-GP4, which was approximately ten times less than that of GP1 (21.2-42.5 $\mu\text{mol/g}$). GP4 had the highest total flavonoid content but the second lowest total quercetin concentration regardless the solvents. It was noted that GP1 had the second highest total flavonoid content and the highest quercetin level in all solvent extracts. Taking together, data in **Table 2.1** indicated that more than two or more solvent systems are required to estimate total phenolic, saponin, and flavonoid. Commercial GP samples may significantly differ in their phenolic, flavonoids, and saponin contents.

Table 2.1. Phytochemical Properties of *G. pentaphyllum**

	Solvents	TPC (mg GAE/g)	TSC (mg GE/g)	TFC (mg RE/g)	Rutin content (μ g/g)	Quercetin content (μ g/g)	R + Q (μ mol QE/g)
GP1	50% acetone	44.3 \pm 0.6j	38.02 \pm 1.10b	21.44 \pm 0.32g	3049.5 \pm 85.7de	4906.5 \pm 105.5d	21.2
	75% ethanol	37.5 \pm 1.0i	41.39 \pm 0.31bc	26.40 \pm 0.05i	7948.2 \pm 282.3g	7431.8 \pm 196.7e	37.6
	100% ethanol	33.6 \pm 0.9h	87.28 \pm 1.37fg	26.87 \pm 0.23i	11235.4 \pm 356.9i	7279.1 \pm 110.0e	42.5
GP2	50% acetone	14.9 \pm 0.3e	90.17 \pm 3.98g	10.16 \pm 0.20c	2527.3 \pm 59.1cd	117.5 \pm 0.8a	4.5
	75% ethanol	12.9 \pm 0.1d	114.48 \pm 0.22i	14.27 \pm 0.17de	3588.1 \pm 610.9e	136.2 \pm 21.9a	6.3
	100% ethanol	6.9 \pm 0.1a	132.57 \pm 0.65k	13.84 \pm 0.14d	2131.9 \pm 73.0c	166.3 \pm 0.5ab	4.0
GP3	50% acetone	12.3 \pm 0.1cd	47.62 \pm 2.14c	10.52 \pm 0.18c	8614.9 \pm 35.0g	358.9 \pm 0.3bc	15.3
	75% ethanol	10.6 \pm 0.3bc	59.13 \pm 3.41d	9.51 \pm 0.10b	9954.0 \pm 173.3h	411.0 \pm 4.3c	17.7
	100% ethanol	6.7 \pm 0.1a	64.57 \pm 1.65d	8.05 \pm 0.05a	7193.0 \pm 24.5f	549.4 \pm 11.6c	13.6
GP4	50% acetone	43.2 \pm 1.2i	77.46 \pm 1.28e	63.48 \pm 0.18l	1409.2 \pm 117.1b	241.3 \pm 2.4ab	3.1
	75% ethanol	30.4 \pm 0.5g	82.12 \pm 1.65ef	54.04 \pm 0.25k	680.2 \pm 2.9a	150.8 \pm 3.9ab	1.6
	100% ethanol	17.7 \pm 0.1f	104.10 \pm 2.54h	36.47 \pm 0.14j	579.4 \pm 42.3a	151.3 \pm 1.8ab	1.4
GP5	50% acetone	13.1 \pm 0.8d	23.61 \pm 1.03a	14.55 \pm 0.13e	ND	ND	
	75% ethanol	10.2 \pm 0.3b	60.70 \pm 1.70d	16.53 \pm 0.10f	ND	ND	
	100% ethanol	8.9 \pm 0.2b	123.97 \pm 3.39j	22.11 \pm 0.22h	ND	ND	

*GP1-5 represent *G. pentaphyllum* samples from different sources. Data are based on per gram of dry botanical and are expressed as mean \pm SD. Different letters represent significant differences ($P < 0.05$). ND stands for not detectable. TPC, TSC, and TFC stand for total phenolic content, total saponin content, and total flavonoid content by spectrometric methods respectively. GAE, GE, RE and QE stand for gallic acid equivalents, gypenoside equivalents, rutin equivalents and quercetin equivalents. Rutin and Quercetin content were flavonoid profile obtained by HPLC. R + Q stands for total amount of flavonoid calculated from rutin and quercetin content.

2.4.4 Relative DPPH Radical Scavenging Capacity (RDSC)

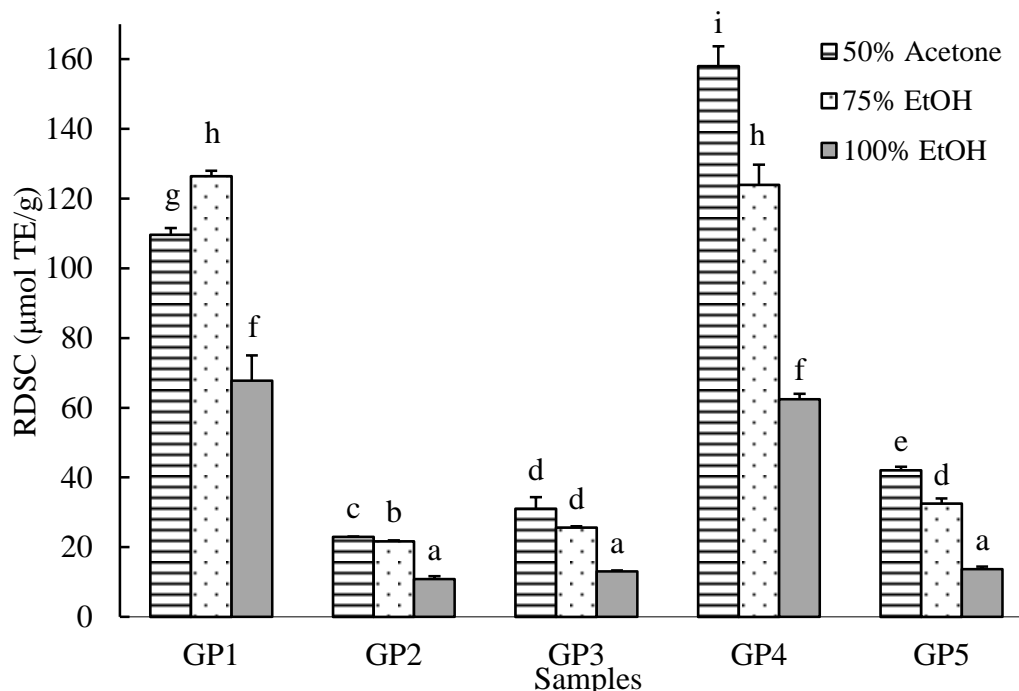


Figure 2.1. Relative DPPH Radical Scavenging Capacity (RDSC) of *G. pentaphyllum* Extracts.

GP1-5 represent *G. pentaphyllum* samples from different sources. 50% Acetone, 75% EtOH, and 100% EtOH represent the 50% acetone, 75% ethanol, and 100% ethanol extracts, respectively. TE stands for the trolox equivalents. The vertical bars represent the standard deviation ($n = 3$) of each data point. Different letters represent significant differences ($P < 0.05$).

Free radical mediated oxidation reactions are crucial for life but may also be damaging. It may cause damage of cellular DNA, protein, and membrane lipid. DNA damage may lead to oncogenic activation (Rakoff-Nahoum, 2006). Also, radical and oxidative stress is closely related to the development of inflammation by activating transcription factors important for the regulation of pro-inflammatory cytokines (Winrow et al., 1993). Natural antioxidants capable of quenching radicals may prevent cellular components from oxidative damage and benefit human health. In the present study, free radical scavenging capacity of *G. pentaphyllum* extracts was investigated against DPPH radicals. The DPPH radical scavenging capacity of the 50%

acetone, 75% ethanol and 100% ethanol extracts of *G. pentaphyllum* are shown in **Figure 2.1**. All the GP extracts showed significant DPPH radical scavenging capacity under the experimental conditions (**Figure 2.1**). The GP samples might differ in their DPPH radical scavenging capacity. GP4 had the highest relative DPPH radical scavenging capacity (RDSC) of 158 μmol trolox equivalent (TE)/g GP botanical sample using 50% acetone as the extraction solvent, followed by that of GP1 at 110 μmol TE/g botanical (**Figure 2.1**). The 100% ethanol extract had the lowest RDSC value and 50% extract had the highest RDSC value for each GP samples, except GP1, suggesting 50% is a preferred solvent for RDSC evaluation for GP samples. The RDSC showed a good correlation ($R = 0.930$, $P < 0.001$) with TPC and an inferior correlation ($R = 0.830$, $P < 0.001$) with TFC, indicating that phenolic compounds might play an important role in the overall radical scavenging capacity of GPs.

The DPPH radical scavenging capacity was further confirmed using the electron spin resonance (ESR) method. ESR determines the presence of unpaired electrons, and is commonly used for free radical examinations (Cheng et al., 2007). The DPPH-GP antioxidant reactions were dose dependent (**Figure 2.2**), whereas no time dependence was observed (data not shown). The ESR analysis also confirmed that the 100% ethanol extract of GP1 had the strongest DPPH scavenging capacity followed by that of GP4 extract.

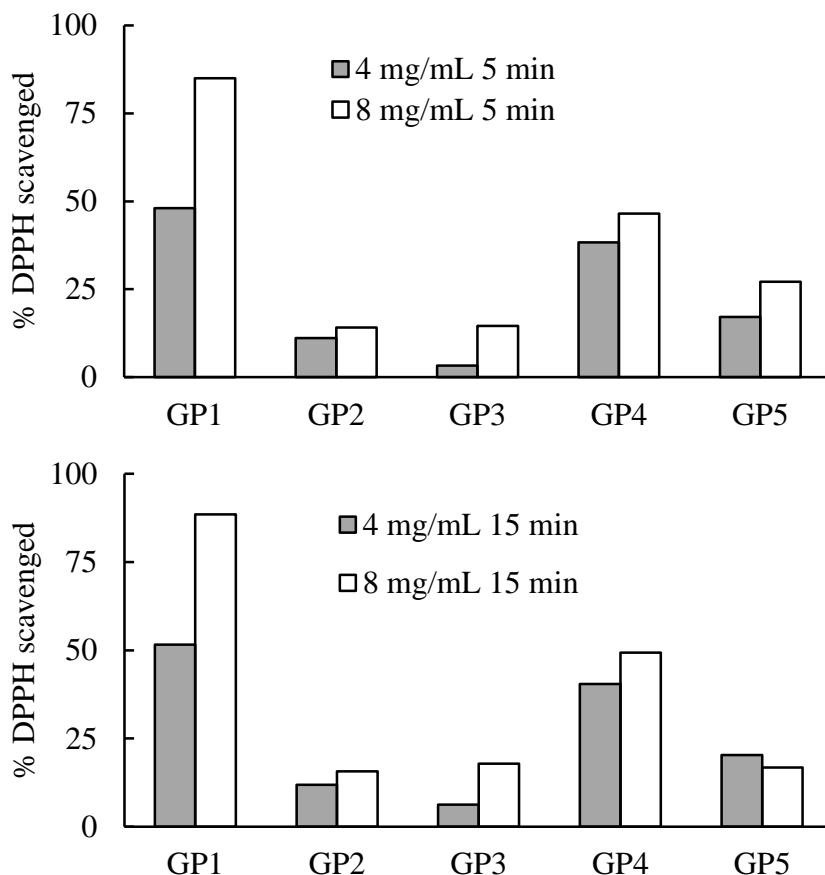


Figure 2.2. DPPH Radical Scavenging Properties of *G. pentaphyllum* Extracts Determined by ESR Method.

GP1-5 represent *G. pentaphyllum* samples from different sources. Control, 4 mg/mL, 8 mg/mL represent the concentration of GP extract at 0, 4, and 8 mg/mL in the reaction mixtures, respectively.

2.4.5 Hydroxyl Radical Scavenging Capacity (HOSC)

Figure 2.3 shows hydroxyl radical scavenging capacity for the 50% acetone, 75% ethanol, and 100% ethanol extracts of *G. pentaphyllum*. To avoid the solvent interference of the assay, solvents in 75% ethanol and 100% ethanol extracts were replaced by 50% acetone prior to the determination. The extracts differed in their HOSC on a per same botanical weight basis though all extracts had significant hydroxyl radical scavenging capacities. The 50% acetone extract showed the greatest HOSC value among the three extracts of GP2-5, but the 75% ethanol extract had the

highest HOSC among the three extracts of GP1, indicating that 50% acetone may serve as a better solvent for HOSC estimation for GP samples. The range of HOSC values for 50% acetone extracts of GP1-GP5 was 162-679 $\mu\text{mol TE/g}$ botanical samples, which was two to five folds higher than those for soybean meal (Slavin et al., 2009). HOSC values were correlated to total phenolic content ($R = 0.940$, $P < 0.001$) and total flavonoid content ($R = 0.800$, $P < 0.001$). Also, HOSC was significantly correlated with the DPPH radical scavenging capacity ($R = 0.954$, $P < 0.001$). These data suggested that the observed antioxidant capacity might mainly result from phenolic/flavonoid components.

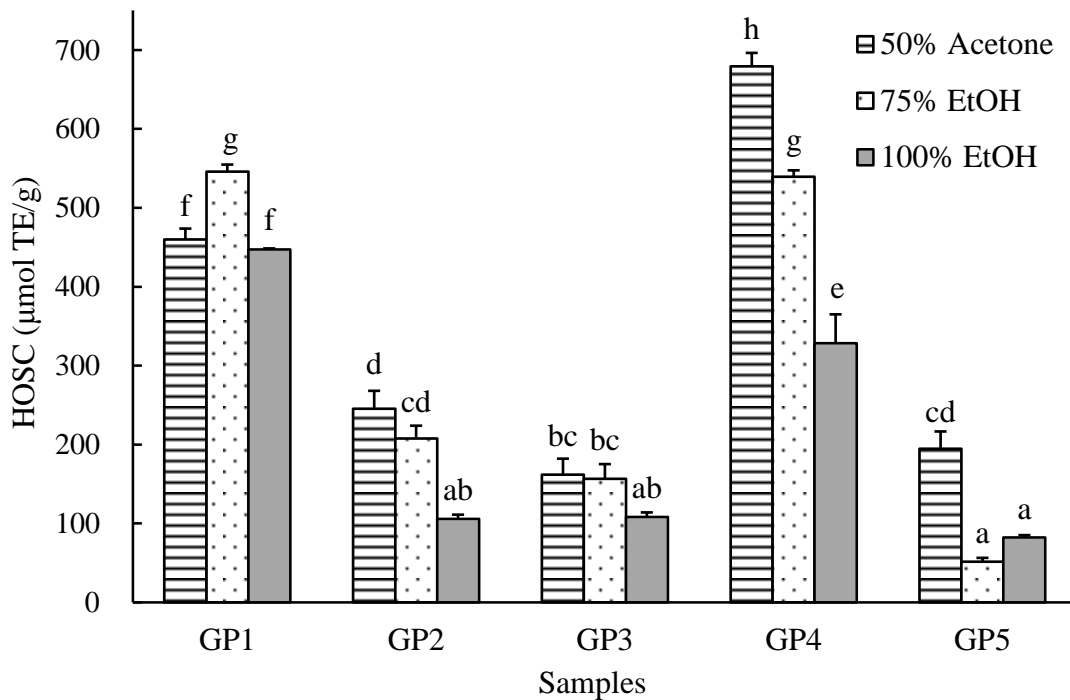


Figure 2.3. Hydroxyl Radical Scavenging Capacity (HOSC) of *G. pentaphyllum* Extracts.

GP1-5 represent *G. pentaphyllum* samples from different sources. 50% Acetone, 75% EtOH, and 100% EtOH represent the 50% acetone, 75% ethanol, and 100% ethanol extracts, respectively. TE stands for the trolox equivalents. The vertical bars represent the standard deviation ($n = 3$) of each data point. Different Letters represent significant differences ($P < 0.05$).

2.4.6 Effects of *G. pentaphyllum* Components on HT-29 Cell Proliferation

The 100% ethanol extracts were re-dissolved in DMSO and used to investigate the anti-proliferative effects of GP components. **Figure 2.4** reports the growing status of HT-29 colon cancer cells in the present of *G. pentaphyllum* extracts at an initial concentration of 3.2 mg botanical equivalents (BE)/mL culture media, whereas no inhibitory effect was observed at 0.032 and 0.32 mg BE/mL concentrations in 96 hours (data not shown). At 3.2 mg BE/mL, GP4 showed the strongest inhibitory effect after 4 hours of treatment. GP1 showed inferior but similar inhibitory behavior. GP2 and GP3 had intermediate suppression during the treatment time, whereas GP5 had slight cell growth suppression capacity. These data suggested that the five GP samples might differ in their anti-proliferative properties.

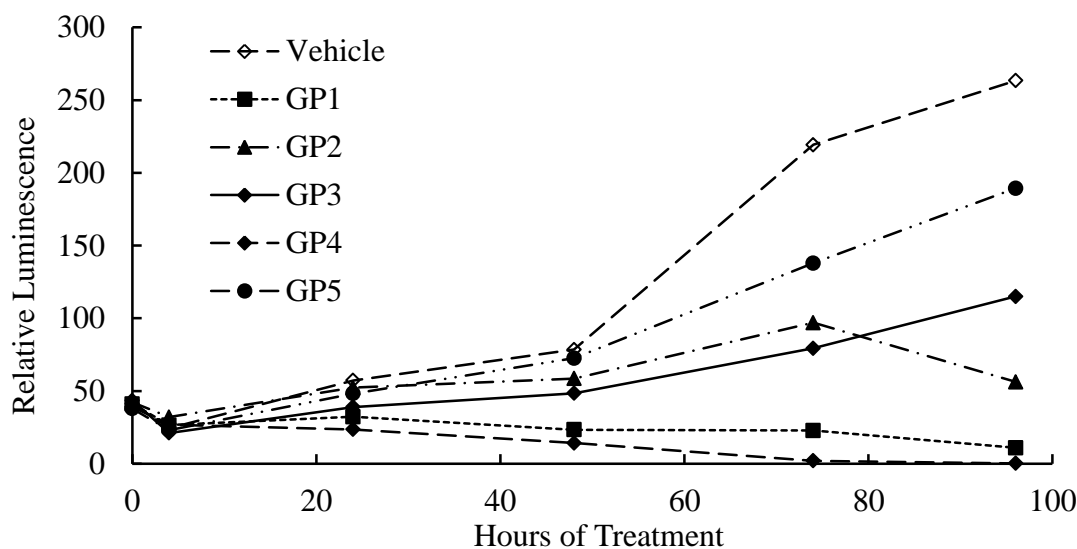


Figure 2.4. Effects of *G. pentaphyllum* Extracts on HT-29 Cell Growth.

GP1-5 represent the five *G. pentaphyllum* samples. 100% ethanol extracts were re-dissolved in DMSO and used in the study. The final concentration of extracts was 3.2 mg botanical equivalents/mL in the initial culture media.

Flavonoids have long been recognized for their potential anti-cancer properties (Havsteen, 2002). This study showed weak inverse correlation between the cell growth (at 72h) and total phenolic content ($R = -0.701$) and total flavonoid content ($R = -0.636$), suggesting that phenolics and flavonoids may contribute to the overall anti-proliferative effect of GP extracts. Saponin content was not closely related to the anti-proliferative effect under the experimental conditions, with a Pearson coefficient factor less than 0.5, although saponins have been related to anti-cancer and anti-inflammatory potentials of *G. pentaphyllum* (Li et al., 1993a; Li et al., 1993b; Lu et al., 2008a; Lu et al., 2008b). Additional research is required to identify the anti-proliferative chemicals in the 100% ethanol extracts of *G. pentaphyllum* and test for their anti-proliferative activities.

2.4.7 Effects of *G. pentaphyllum* Extracts on TNF- α , IL-6 and Ptgs2 mRNA Expression

The effects of GP extracts on mRNA expression were measured in LPS-stimulated mouse J774A.1 macrophage cells. LPS-stimulated macrophage cells were employed to simulate pro-inflammatory response in vivo. As shown in **Figure 2.5**, GP1-5 extracts at the initial concentration of 3.2 mg extract/mL significantly suppressed the LPS-induced TNF- α mRNA expression, whereas GP1, GP2, GP4 and GP5 at 0.32 mg extract/mL concentration had significantly inhibited the LPS stimulated TNF- α mRNA expression. GP1 extract had the strongest inhibitory effect on TNF- α mRNA expression among all five samples. Dose-dependent manner was only observed for GP4 extract under the experimental conditions.

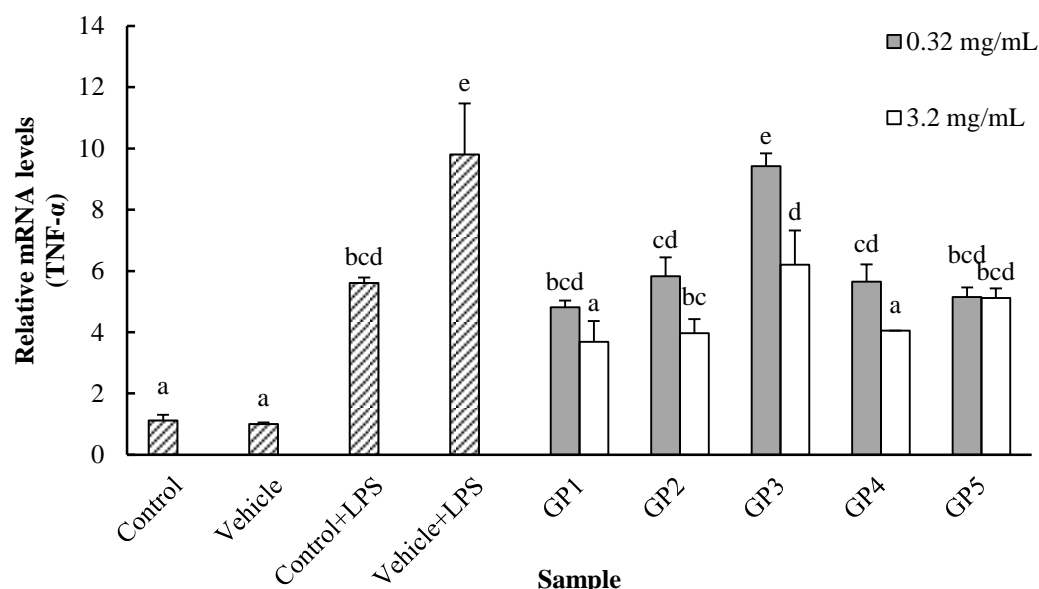


Figure 2.5. Effects of Ethanol Extracts of *G. pentaphyllum* on TNF- α mRNA Expressions in Mouse J774A.1 Macrophage Cells.

GP1-5 represent *G. pentaphyllum* samples from different sources. The final concentrations of extracts were 0.32 and 3.2 mg botanical equivalents/mL in the initial culture media. The vertical bars represent the standard deviation ($n = 3$) of each data point. Different letters represent significant differences ($P < 0.05$).

The IL-6 and Ptgs2 mRNA expressions in the LPS-stimulated mouse J774A.1 macrophage cells were completely inhibited at 3.2 mg botanical equivalents/mL concentration (**Figure 2.6 and 2.7**). The experiment data also indicated that DMSO may further stimulate the expression of TNF- α , IL-6 and Ptgs2 mRNA in macrophage cells in the presence of LPS, while DMSO has little stimulating effect without the presence of LPS.

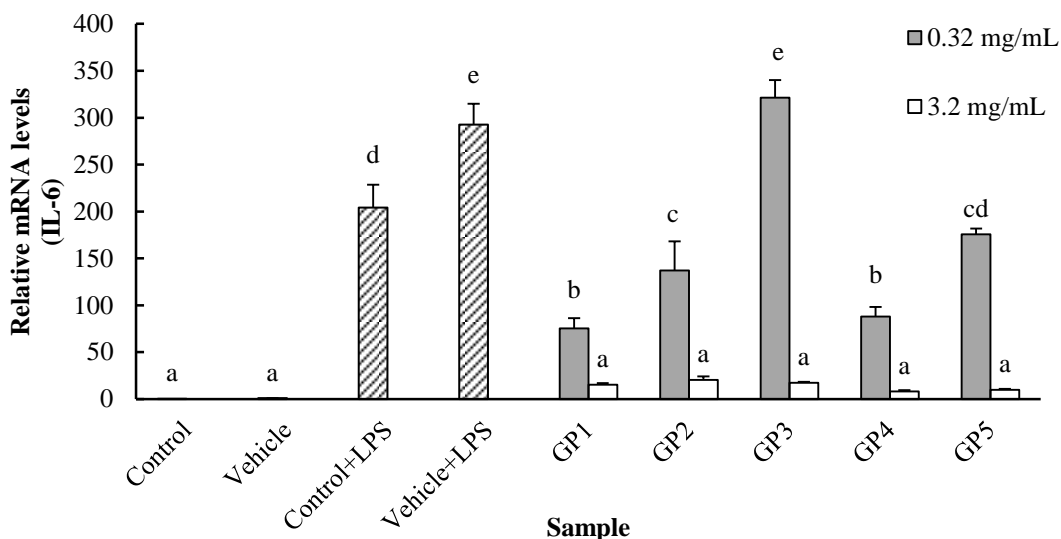


Figure 2.6. Effects of Ethanol Extracts of *G. pentaphyllum* on IL-6 mRNA Expressions in Mouse J774A.1 Macrophage Cells.

GP1-5 represent *G. pentaphyllum* samples from different sources. The final concentrations of extracts were 0.32 and 3.2 mg botanical equivalents/mL in the initial culture media. The vertical bars represent the standard deviation (n = 3) of each data point. Different letters represent significant differences ($P < 0.05$).

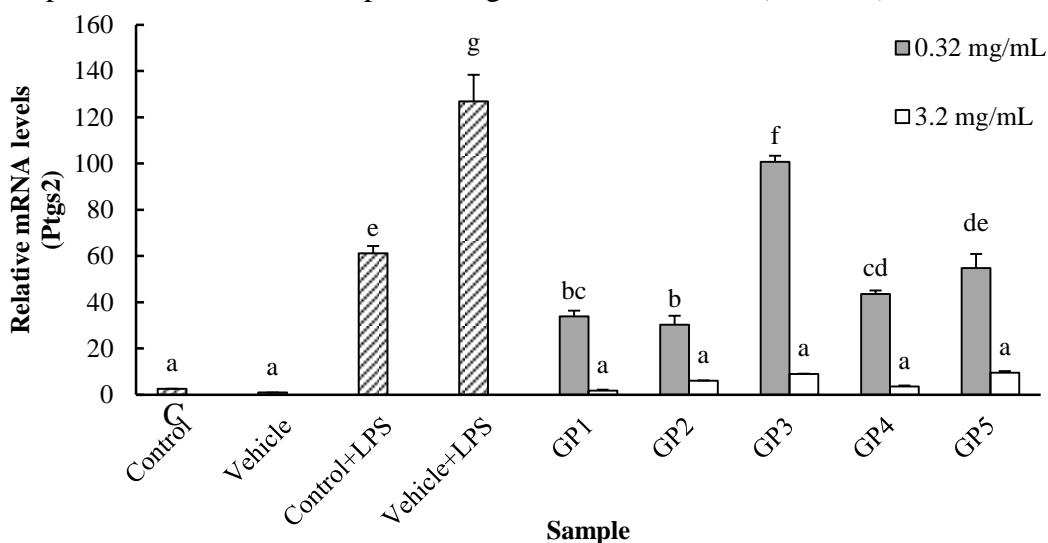


Figure 2.7. Effects of Ethanol Extracts of *G. pentaphyllum* on Ptgs2 mRNA Expressions in Mouse J774A.1 Macrophage Cells.

GP1-5 represent *G. pentaphyllum* samples from different sources. The final concentrations of extracts were 0.32 and 3.2 mg botanical equivalents/mL in the initial culture media. The vertical bars represent the standard deviation (n = 3) of each data point. Different letters represent significant differences ($P < 0.05$).

2.4.8 Water Content, Fiber Content and Selenium Concentration

Water content, fiber content and selenium concentration of the five GP samples were determined. The water content ranged from 3.79 to 7.57 g/100 g of sample, and the differences between samples could be significant. Dietary fiber is vital in human's diet and has many possible health effects such as reduced risk of colon cancer (Bingham, Day, Luben, Ferrari, Norat, Slimani et al., 2003), and heart disease and hyperglycemia (Wolever, 1990). GP3 had the highest total fiber content of 0.6 g/g and GP5 had the lowest total fiber content of 0.24 g per gram of *G. pentaphyllum*. To our best knowledge, this is the first report of total fiber content of GP botanicals. Selenium content was determined for the five GP samples since GP1, GP2, and GP3 were grown in a selenium rich area. The highest Se content of 1.7 mg/g was detected in GP3 under the experimental conditions. The GP samples grown in Se rich soil may not necessarily have a greater Se content (**Table 2.2**). Selenium is an essential micronutrient for humans and its impact on human health is a global topic of interest.

Table 2.2. Water Content, Fiber Content and Selenium Concentration of *G.pentaphyllum**

	Water Content (g/100 g)	Fiber content (g/100 g)	Selenium Concentration (mg/kg)
GP1	5.36 ± 0.19b	41.77	0.229
GP2	3.79 ± 0.15a	34.41	0.138
GP3	5.54 ± 0.07b	60.45	1.730
GP4	7.57 ± 0.06c	46.24	0.265
GP5	6.70 ± 0.07d	23.87	0.094

*GP1-5 represent *G. pentaphyllum* samples from different sources. Data are based on dry botanical. Data of water content are expressed as mean ± SD. Different letters represent significant differences ($P < 0.05$).

In summary, this study demonstrated that *G. pentaphyllum* is rich in flavonoids and saponins, and may have significant levels of antioxidant, anti-proliferative and anti-inflammatory components. Results also suggest that individual *G. pentaphyllum* grown from different locations may significantly differ in their phytochemical composition and nutraceutical properties. In addition, solvent selection may alter the estimation of phytochemical composition and content, and health properties of *G. pentaphyllum*.

Chapter 3: Chemical Composition of Five Commercial *Gynostemma pentaphyllum* Samples and Their Radical Scavenging, Anti-proliferative, and Anti-inflammatory Properties

Xie, Z.; Huang, H.; Zhao, Y.; Shi, H.; Wang, S.; Wang, T. T. Y.; Chen, P.; Yu, L. (2012). *Food Chem.* 132, 125-133

3.1 Abstract

Leaf and whole-plant samples of the diploid and tetraploid *Gynostemma pentaphyllum* (GP) were investigated and compared for their chemical compositions, and their potential anti-proliferative and anti-inflammatory effects. The highest levels of total flavonoids and phenolics were observed in the diploid leaf botanical (2L3) at 36.84 mg rutin equiv/g and 41.15 mg gallic acid equiv/g, respectively. The diploid leaf sample (2L2) had highest amount of rutin and quercetin contents of 77.7 μ mol quercetin equiv/g. The tetraploid whole-plant botanical (4L3) had the highest total saponin content of 227.1 mg gypenoside equivalents/g. Extracts from all tested GP samples showed time- and dose-dependent antiproliferative effects in HT-29 cells, and diploid leaf samples had the overall highest inhibitory activity. These extracts had different order of antiproliferative properties in the LNCaP cells, suggesting the potential selective inhibition of GP extracts against different types of cancer cells and the effect of cell model in screening and evaluation of antiproliferative components. In addition, the diploid leaf extracts showed the strongest inhibitory effects on expression of TNF- α , IL-6 and COX-2 mRNA at final concentrations of 0.2 and 1 mg

botanical equivalents/mL media. The results from this study will be used to develop new nutraceutical products from *Gynostemma pentaphyllum*.

3.2 Introduction

Gynostemma pentaphyllum, also known as Jiaogulan, has been traditionally used as food and tea (Wang et al., 2007). In the past decades, the consumption of *G. pentaphyllum* Makino, a perennial liana plant, has been steadily increased since growing evidence indicated that *G. pentaphyllum* may potentially reduce the risk of cardiovascular diseases (Tanner et al., 1999), and may have hypoglycemic (Yeo et al., 2008), anti-inflammatory (Lin et al., 1993), anticancer (Chen et al., 2009a; Lu et al., 2008b) and hepatoprotective activities (Chen et al., 2009b) with little concern of toxic effects (Attawish et al., 2004; Schild et al., 2010). Flavonoids and gypenosides, the saponins of *G. pentaphyllum*, are believed as the primary phytochemicals contributing to the health properties (Wang et al., 2007). *G. pentaphyllum* is grown in several provinces in China and marketed in several countries including the United States, and becomes an important source of agricultural income in some regions.

It is well recognized that the phytochemical composition and health properties of botanical materials may vary greatly because of inherent factors such as genotype, and external factors such as growing environment and post-harvest treatments and the interactions between these factors (Moore et al., 2006a; Whent et al., 2009). Genotype is considered one of the most important factors in determining the chemical compositions in botanicals. Polyploidization, a common method for genetic modification, is an effective way to alter chemical properties and may yield a desirable plant genotype. For example, wheat with different chromosome ploidy

performed differently in drought tolerance and yield performance (Xiong et al., 2006). It was also noted that tetraploid ginger had much higher total gingerol content than its diploid counterpart (Sanwal et al., 2010). Our recent study has shown significant amount of phenolics, flavonoids, saponins and anti-proliferative and anti-inflammatory effect of five commercial *G. pentaphyllum* samples (Xie, Liu, Huang, Slavin, Zhao, Whent et al., 2010b). To our knowledge, previous researches on *G. pentaphyllum* were only related to diploid GP samples, and chemical compositions and biological activities of polyploid plant of *G. pentaphyllum* have not been explored or systematically investigated.

As a continuation of our previous study on *G. pentaphyllum*, sixteen samples of different parts from different genotypes (diploids vs tetraploids) of *G. pentaphyllum* were examined and compared for their total phenolic, total saponin, total flavonoid contents, rutin and quercetin contents, and their scavenging capacities against DPPH and hydroxyl radicals. Also investigated were their anti-proliferative activity against HT-29 human colon cancer cells and LNCaP human prostate cancer cells, and potential anti-inflammatory effects. The anti-inflammatory effects were determined as their capacities to suppress TNF- α , IL-6 and COX-2 gene expression in cultured Mouse J774A.1 macrophages. The results from this study will be used to promote the better production and use of *G. pentaphyllum* for improving human health.

3.3 Materials and Methods

3.3.1 Materials

Sixteen *G. pentaphyllum* Makino samples were obtained from the Asian Citrus Holdings Limited (Hong Kong), including four diploid leaf samples (2L1-2L4), four diploid whole-plant samples (2W1-2W4), four tetraploid leaf samples (4L1-4L4), and four tetraploid whole-plant samples (4W1-4W4). The whole-plant sample included both the leaf and stem of the plant. All botanical samples were grown in the Dabashan area of Shaanxi province of China in 2009, and harvested by hands from the different locations of the same field according to a standard randomization protocol. The samples were washed using tap water, dried in a tea drier at 70-76 °C, pulverized with a conventional pulverizing machine, and kept in zip lock bags in dark until analysis. The ploidy was determined by root tip chromosome checking (22 vs. 44).

Iron (III) chloride, fluorescein (FL), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH[•]) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) were obtained from Sigma-Aldrich (St. Louis, MO). 2,2'-azinobis (2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals USA (Richmond, VA). Thirty percent ACS-grade H₂O₂ was purchased from Fisher Scientific (Fair Lawn, NJ). The Mouse J774A.1 macrophage cell line was obtained from ATCC (Manassas, VA). Lipopolysaccharide (LPS) was purchased from Sigma-Aldrich (St. Louis, MO). Ultrapure water was prepared by an ELGA Purelab ultra Genetic polishing system with < 5 ppb TOC and resistivity of 18.2 mΩ (Lowell, MA) and was

used for all experiments. The cell culture media were purchased from Invitrogen (Carlsbad, CA). All other chemicals and solvents were of the highest commercial grade and used without further purification.

3.3.2 Testing Sample Preparation

Botanical samples were ground to a particle size of 40-mesh using a household coffee grinder. Approximately twelve grams of ground samples were extracted in pure ethanol for 5 h using a Soxhlet apparatus. Extracts were kept in dark until testing.

3.3.3 Determination of Se Concentration

Se concentration was determined by inductively coupled plasma dynamic reaction cell mass spectrometry (ICP-DRC-MS) in a commercial analytical lab (Applied Speciation and Consulting LLC, Bothell, WA).

3.3.4 Moisture Content

The moisture content was measured by drying samples in an oven at 100°C for four hours. The weight loss after drying was used to calculate the moisture content.

3.3.5 Fiber Content

Soluble and insoluble fiber contents were measured with a commercial kit purchased from Megazyme International Ireland Ltd. (Wicklow, Ireland) according to the previously reported procedure (Yu et al., 2003). Equal aliquot of the 4 samples of diploid leaf was combined to represent diploid leaf of *G. pentaphyllum* (2L).

Likewise, 4 samples of diploid whole plant (2W), tetraploid leaf (4L), and tetraploid whole plant (4W) were combined and subjected to the tests, respectively.

3.3.6 Total Phenolic Content (TPC)

TPC of the extraction was measured according to a laboratory procedure described previously (Yu et al., 2002). Briefly, 50 μ L of sample extract or standard was mixed with 250 μ L of the Folin-Ciocalteu reagent, 750 μ L of 20% sodium carbonate, and 3 mL of ultrapure water. Gallic acid was used as the standard. Absorbance was read at 765 nm on a Genesys 20 spectrophotometer (Thermo Scientific, Waltham, MA) after 2 hours of reaction at ambient temperature in dark. Reactions were conducted in triplicate and results were reported as mg gallic acid equivalents (GAE) per gram of the botanical sample.

3.3.7 Total Flavonoid Content (TFC)

Total flavonoid content was determined by an aluminum colorimetric method described previously (Xie et al., 2010b). Briefly, the sample extract (150 μ L) was mixed with 1 mL of 5% sodium nitrite. After 6 min, 1 mL of 10% aluminium nitrate was added. Then 4 mL of 4% sodium hydroxide was added into the mixture. Rutin was used as the standard. Absorbance was read at 502 nm on a Genesys 20 spectrophotometer (Thermo Scientific, Waltham, MA) after 15 min of reaction. The results were reported as mg rutin equivalents per gram of botanicals.

3.3.8 Total Saponin Content (TSC)

The total saponin content was determined using the method described previously (Xie et al., 2010b). Briefly, the extract (100 μ L) was evaporated by nitrogen and mixed with 200 μ L of 5% vanillin in acetic acid and 800 μ L of 70% perchloric acid. The mixture was incubated at 60 $^{\circ}$ C for 15 min. A commercial *G. pentaphyllum* gypenoside extract was used as the quantification standard. Glacial acetic acid (5 mL) was added into the mixture before the absorbance was read at 550 nm on a Genesys 20 spectrophotometer (Thermo Scientific, Waltham, MA) after sample cooled down to ambient temperature.

3.3.9 Rutin and Quercetin Contents

The rutin and quercetin contents were determined by HPLC-DAD analysis according to the laboratory protocol described previously (Xie et al., 2010b). The ground samples were extracted in methanol for 6 h using a Soxhlet apparatus and then filtered through 0.45 μ m membrane filters. Compounds were separated on an Agilent Eclipse Plus C18, 150 \times 2.1 mm, 1.8 μ m column at a flow rate of 0.25 mL/min. The HPLC separation was accomplished using a binary gradient system. Briefly, mobile phase A consisted of 0.1% formic acid (FA) in H₂O and mobile phase B consisted of 0.1% FA in acetonitrile. The initial ratio of A to B was 90:10 (v/v); this was changed linearly to 55:45 (v/v) in 20 min, to 10:90 (v/v) after 30 min, and kept at 10:90 (v/v) from 30 min to 40 min. The wavelength for UV detection was 256 nm. The flavonoids were identified via comparison to standards for retention time and UV spectrum, and were quantified via the area under the curve using external standards.

3.3.10 Relative DPPH Radical Scavenging Capacity (RDSC)

The RDSC values were determined following a laboratory procedure described previously (Cheng et al., 2006). Briefly, 100 μL of sample extract, solvent, or the standard solution of trolox was added to 100 μL of freshly prepared DPPH^\bullet solution to initiate antioxidant-radical reaction. The absorbance of the reaction mixture was measured at 515 nm every minute during 40 min of reaction. An initial DPPH^\bullet concentration of 100 μM was used for all reactions. RDSC values were calculated using areas under the curve relative to trolox standards. Results are expressed as micromoles of trolox equivalents (TE) per gram of botanical sample.

3.3.11 Hydroxyl Radical Scavenging Capacity (HOSC)

HOSC assay was conducted according to a previously reported laboratory protocol using a Victor³ multilabel plate reader (PerkinElmer, Turku, Finland) (Moore et al., 2006b). Standard solutions of trolox were prepared in 50% acetone. Reaction mixtures consisted of 170 μL of 9.28×10^{-8} M FL prepared in 75 mM sodium phosphate buffer, 30 μL of standard, sample extract or blank, 40 μL of 0.1990 M H_2O_2 , and 60 μL of 3.43 mM FeCl_3 . Fluorescence was measured every minute for 3 h with an excitation wavelength of 485 nm and an emission wavelength of 535 nm. HOSC values were expressed as micromoles of trolox equivalents (TE) per gram of sample.

3.3.12 HT-29 Colon Cancer Cell Proliferation Inhibition

Equal aliquots of 100% ethanol extracts from the 4 samples of diploid leaf were combined to make a new extract (2L). Likewise, 4 samples of diploid whole

plant, tetraploid leaf, and tetraploid whole plant samples were made into new extracts labeled as 2W, 4L, and 4W, respectively. After removal of the solvent, the extracts were re-dissolved in DMSO, and the final concentrations of *G. pentaphyllum* in cell media were 0, 1, 10 mg of botanical equiv/mL with 0.3% DMSO. Botanical equivalent was calculated as the original plant material equivalent. HT-29 human colorectal adenocarcinoma cell proliferation inhibition was investigated according to a previously reported protocol (Slavin et al., 2009). The cells were grown at 37 °C under 5% carbon dioxide in McCoy's 5A medium supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic. An ATP-Lite 1 step kit (Perkin-Elmer Life and Analytical Sciences, Shelton, CT) was used to determine cell proliferation. The emitted luminescence was determined using a Victor³ multiwell plate reader (Perkin-Elmer, Turku, Finland) immediately prior to treatment and at 4, 24, 48, 72, and 96 h after initial treatment. Treatment media were replaced every 24 h until a reading was taken on that plate.

3.3.13 LNCaP Prostate Cancer Cell Proliferation Inhibition

The anti-proliferation study was performed according to a previously described protocol (Wang, Hudson, Wang, Remsberg, Davies, Takahashi et al., 2008). LNCaP cells (5×10^5 cells per well) were plated in 6-well plates (Costar, Corning Incorporated, Corning, NY). Cells were incubated in a humidified atmosphere at 37 °C and 5% carbon dioxide, in a culture media of RPMI media supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic for 24 h, and then cells were treated with samples with DMSO as the vehicle. In the media, the concentrations of the *G. pentaphyllum* were 20, 100, 200 and 1000 µg botanical

equivalents /mL, and fresh medium with samples were replaced every 24 h. Cell growth was analyzed using a Sulforhodamine B (SRB) assay (Takahashi, Perkins, Hursting, & Wang, 2007).

3.3.14 Effects on TNF- α , IL-6 and COX-2 Gene Expression in Mouse J774A.1 Macrophage Cells

The DMSO solution was used for anti-inflammatory study, and the final concentrations of *G. pentaphyllum* in cell media were 0, 0.2, 1 mg botanical equiv/mL. Mouse J774A.1 macrophages were cultured in 12-well plates overnight and reached 80% confluence. The cells were first treated with cell media containing *G. pentaphyllum* extracts for 2 h. Then lipopolysaccharide (LPS) was added at the initial concentration of 0.5 μ g/mL. Cells were incubated at 37 $^{\circ}$ C and 5% CO₂ for 24 h.

The cells were used for RNA isolation and Real-time PCR analysis. RNA isolation and Real-time PCR of TNF- α , IL-6 and Ptg2 were performed following the previously reported protocol (Trasino et al., 2009; Zhou et al., 2009). After 24 h incubation, the TRIzol reagent (Invitrogen) was used for total RNA isolation, and StrataScript First Strand complementary DNA Synthesis kit (Stratagene) was used to reverse transcribe complementary DNA. Real-time PCR was performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using TaqMan Universal PCR Master Mix. The TaqMan Assay-On-Demand was purchased from Applied Biosystems: Il6 (Mm00446190), Tnf (Mm00443258), Ptg2 (Mm01307329). The mRNA contents were normalized to an internal control, Tbp (Mm00446973) mRNA. The following amplification parameters were used for PCR: 50 $^{\circ}$ C for 2 min,

95 °C for 10 min, and 46 cycles of amplification at 95 °C for 15 s and 60 °C for 1 min.

3.3.15 Statistic Analysis

Data were reported as mean \pm SD for triplicate determinations on an ‘as-it-is’ botanical weight basis. One-way ANOVA and Tukey’s test were employed to identify differences in means. Statistics were analyzed using SPSS for Windows (version rel. 10.0.5, 1999, SPSS Inc., Chicago, IL). Correlation was analyzed using a two-tailed Pearson’s correlation test. Statistical significance was declared at $P < 0.05$.

3.4 Results and Discussion

3.4.1 Total Phenolic Content (TPC)

Table 3.1. Phytochemical Properties and Moisture Content of *G. pentaphyllum* *

	TPC (mg GAE/g)	TFC (mg RE/g)	TSC (mg GE/g)	R+Q (μ mol QE/g)	Se (mg/kg)	moisture content (g/100 g)
2L1	33.65 \pm 1.60gh	30.62 \pm 0.53f	102.04 \pm 3.04b	63.0 \pm 0.2m	1.38	4.24
2L2	35.67 \pm 3.79h	27.07 \pm 0.38e	93.44 \pm 3.27b	77.7 \pm 0.6p	1.34	5.65
2L3	41.15 \pm 1.98i	36.84 \pm 0.82h	123.87 \pm 5.74c	75.6 \pm 0.2o	1.62	5.09
2L4	22.48 \pm 0.47d	19.89 \pm 0.16d	71.64 \pm 1.78a	71.1 \pm 0.7n	1.56	5.42
Mean _{2L}	33.24 \pm 7.36x	28.61 \pm 6.42x	98.14 \pm 20.70z	71.8 \pm 6.0w	1.48	5.10
2W1	15.40 \pm 0.51c	17.46 \pm 0.30b	68.32 \pm 0.82a	38.5 \pm 0.0k	1.36	1.82
2W2	13.80 \pm 0.26c	18.40 \pm 0.32bc	100.51 \pm 2.09b	26.9 \pm 0.0j	1.25	5.06
2W3	12.63 \pm 0.62bc	21.09 \pm 0.25d	140.12 \pm 2.52d	16.7 \pm 0.1e	1.09	1.68
2W4	24.09 \pm 0.57de	19.66 \pm 0.21cd	61.00 \pm 5.04a	56.7 \pm 0.0l	1.58	1.34
Mean _{2W}	16.48 \pm 4.72y	19.15 \pm 1.44y	88.16 \pm 30.53z	34.7 \pm 15.9x	1.32	2.47
4L1	30.08 \pm 0.66fg	31.26 \pm 0.53fg	171.16 \pm 7.37e	22.5 \pm 0.0i	4.41	1.74
4L2	27.05 \pm 0.61ef	28.08 \pm 0.94e	178.28 \pm 11.86e	18.6 \pm 0.1f	2.49	5.44
4L3	28.35 \pm 0.39f	31.19 \pm 0.59fg	227.09 \pm 9.31f	21.5 \pm 0.0h	1.40	1.62
4L4	28.33 \pm 0.95f	32.38 \pm 0.94g	211.99 \pm 4.36f	19.8 \pm 0.1g	6.47	2.76
Mean _{4L}	28.45 \pm 1.27x	30.73 \pm 1.80x	198.24 \pm 25.96y	20.8 \pm 1.6y	3.69	2.89
4W1	9.11 \pm 0.11ab	11.96 \pm 0.20a	98.73 \pm 2.04b	8.1 \pm 0.0c	3.06	1.35
4W2	14.75 \pm 0.58c	20.30 \pm 0.30d	149.23 \pm 0.67d	13.4 \pm 0.1d	1.86	1.75
4W3	8.99 \pm 0.22ab	11.60 \pm 0.22a	99.25 \pm 4.17b	4.0 \pm 0.0a	3.02	1.49
4W4	8.07 \pm 0.11a	10.60 \pm 0.30a	88.58 \pm 1.20b	6.8 \pm 0.0b	1.10	0.57
Mean _{4W}	10.23 \pm 2.78z	13.61 \pm 4.07z	105.28 \pm 22.33z	8.7 \pm 3.9z	2.26	1.29

*2L1-2L4 represent four diploid leaf samples; 2W1-2W4 represent four diploid whole-plant (leaf and stem) samples; 4L1-4L4 represent four tetraploid leaf samples and 4W1-4W4 represent four tetraploid whole-plant (leaf and stem) samples. Data are on an 'as-it-is' botanical weight basis and 2L1-2L4, 2W1-2W4, 4L1-4L4 and 4W1-4W4 are expressed as mean \pm SD (n = 3), whereas means of each group are expressed as mean \pm SD (n = 12). Different letters (a-i) represent significant differences among individual samples within a column, while letters x-z indicate difference among the average values of the four samples for each sample type ($P < 0.05$). TPC, TSC, and TFC stand for total phenolic content, total saponin content, and total flavonoid content respectively. R + Q stands for total amount of rutin and quercetin. GAE, RE, GE and QE stand for gallic acid equivalents, rutin equivalents, gypenoside equivalents and quercetin equivalents.

Ethanol extracts of *G. pentaphyllum* were examined for their total phenolic contents. The TPC values ranged from 22.5 to 41.2 mg GAE/g botanicals for diploid leaf samples (2L1-2L4), 12.6-24.1 mg GAE/g for diploid whole-plant samples, 28.3-30.1 mg GAE/g for tetraploid leaf samples and 8.1-14.8 mg GAE/g for tetraploid whole-plant samples (**Table 3.1**). The highest TPC value of 41.2 mg gallic acid

equivalents (GAE)/g *G. pentaphyllum* (GP) botanical was detected in the diploid leaf sample 2L3, and followed by that of 35.7 mg GAE/g in the diploid leaf sample 2L2 (**Table 3.1**). The TPC values ranged from 8.1 to 41.2 mg GAE/g in the present study. This TPC range was comparable to that of 6.7-33.6 mg GAE/g observed in the five commercial *G. pentaphyllum* reported in a previous study (Xie et al., 2010b), suggesting that both diploid and tetraploid samples contained significant amount of phenolic compounds.

It was notable that the TPC values varied significantly among these sixteen samples. The leaf samples had higher TPC values than their counterpart whole-plant samples, regardless of the genotype (**Table 3.1**). Also noted was that TPC values of diploid samples were equal to or greater than that of their tetraploid counterpart samples for both leaf and whole-plant GP samples (**Table 3.1**). These data suggested that phenolic compounds might be concentrated in the leaf for both diploid and tetraploid GP. In addition, TPC values might vary significantly for GP samples with same genotype from different locations (**Table 3.1**). For instance, the highest TPC value of diploid leaf samples was 41.15 mg GAE/g for sample 2L3. This value was about 2 times of the phenolic level of 2L4 (22.48 mg GAE/g). The variation of TPC value was also observed for diploid whole-botanical samples with the highest value of 24.09 mg GAE/g compared to the lowest content of 12.63 mg GAE/g, and for the tetraploid GP whole-botanical samples with the high value of 14.75 mg GAE/g compared to the low content of 8.07 mg GAE/g. This variation might be due to the growing environment and the interaction between genotype and environmental conditions (Whent et al., 2009). In 2009, Whent and others (2009) reported that

genotype and the interaction between genotype and environment determined 40.64% and 53.58% phenolic contents in soybeans grown in Maryland, respectively, while environment only contributed 5.78% influence of soybean TPC. In contrast, environment attributed 79.54% of TPC in hard winter wheat grown in Colorado, whereas genotype and the interaction between genotype and environment only attributed 5.35 and 15.00%, respectively (Moore et al., 2006a; Whent et al., 2009). It would be interesting to know how the genotype, environment, and the interaction between genotype and environment may alter the TPC and other health components and properties in diploid and tetraploid *G. pentaphyllum*.

3.4.2 Total Flavonoid Content (TFC) and Rutin and Quercetin Contents

G. pentaphyllum is well known for its high flavonoid content and significant levels of rutin and quercetin (Xie et al., 2010b). As shown in **Table 3.1**, The TFC ranged 10.6-36.8 mg rutin equivalents (RE)/g of botanical. These values were comparable to that of 8.1-36.5 mg RE/g observed in the five commercial GP samples using ethanol as the extraction solvent in a previous study (Biasioli, Gasperi, Aprea, Colato, Boscaini, & Märk, 2003). The diploid leaf sample 2L3 had the highest total flavonoid content of 36.8 mg RE/g among the sixteen GP samples. The second greatest TFC value of 32.4 mg RE/g was detected in the tetraploid GP leaf sample 4L4 (**Table 3.1**). The diploid leaf samples had a flavonoid content of 19.9-36.8 mg RE/g; the diploid whole-plant samples had a level of 17.5-21.1 mg RE/g; the tetraploid leaf samples had a level of 28.1-32.4 mg RE/g; and the tetraploid whole-plant samples had a level of 10.6-20.3 mg RE/g respectively. The mean TFC values were 28.61, 19.15, 30.73, and 13.61 mg RE/g, for leaf and whole-plant samples of the

diploid and tetraploid GPs, respectively (**Table 3.1**). These data indicated that leaf contained greater amount of flavonoids than the stems regardless of GP genotype.

It was also noted that the leaf or whole-plant samples from different growing locations might differ in their TFC, suggesting the potential effect of growing environment, and the interaction between genotype and environment on flavonoids contents in GP. In addition, significant correlation was detected between the total phenolic content and total flavonoid content in GP samples ($R = 0.918$, $P < 0.0001$), suggesting the significant amount of flavonoids in the total phenolics.

The rutin and quercetin contents were examined by HPLC analysis (**Table 3.1**). Because rutin is the glycoside of quercetin and rutin could be converted to quercetin in vivo, the rutin content was converted into quercetin equivalent (QE) by amount of substance. Therefore the QE concentration represents the total amount of rutin and quercetin. The 16 samples differed in their QE concentrations. The highest value was obtained by the diploid leaf botanical (2L2) at 77.7 $\mu\text{mol QE/g}$, followed by 2L3 at 75.6 $\mu\text{mol QE/g}$. The mean quercetin equivalent values were 71.8, 34.7, 20.8, and 8.7 $\mu\text{mol QE/g}$, for leaf and whole-plant samples of the diploid and tetraploid GPs, respectively.

3.4.3 Total Saponin Content (TSC)

Kao and others (2008) detected and quantified 33 gypenosides which ranged from 0.5 to 89.9 mg/g in *G. pentaphyllum* by HPLC-ELSD-MS. As shown in **Table 3.1**, TSC ranged from 61.0 to 227.1 mg of GE/g in these sixteen samples. These values were comparable to that of 64.6-132.6 mg gypenoside equivalents(GE)/g for

the five commercial GP samples with ethanol as the extraction solvent in a recent study (Xie et al., 2010). The highest value of 227.1 mg of GE/g was observed in the tetraploid leaf sample 4L3, which was followed by 212.0 mg of GE/g in tetraploid 4L4 GP. The lowest value was observed in diploid whole-plant sample 2W4 at 61.0 mg GE/g, and the second lowest TSC of 68.32 mg GE/g was detected in another whole plant diploid sample 2W1. It was noteworthy that tetraploid leaf samples had the highest mean TSC level of 198.24 mg GE/g, which was approximately two times as much as that in the leaf and whole-plant diploid and the whole-plant tetraploid GP samples (**Table 3.1**). The whole-plant diploid GP was not significantly different to the whole-plant tetraploid or leaf diploid GP in TSC.

The GP samples from the same part of same genotype might also differ in their TSC. For instance, TSC value of 2W3 was 2.3 times higher than that of 2W4, both diploid whole-plant samples, implying that growing conditions may play an important role in saponin contents in GP besides genotype. Several studies have indicated saponins might potentially contribute to anti-cancer (Liu et al., 2009) and anti-inflammatory effects of *G. pentaphyllum* (Park et al., 2009). Taken together, these data indicated that tetraploid leaf samples had the highest TSC levels superior to samples from other groups, and may serve as an excellent dietary source for GP saponins.

3.4.4 Relative DPPH Radical Scavenging Capacity (RDSC)

The DPPH radical scavenging capacities of the sixteen *G. pentaphyllum* samples are shown in **Figure 3.1**. A higher RDSC value corresponds to a stronger antioxidant capacity. Diploid leaf sample 2L3 had the greatest RDSC of 68.6 μmol of

trolox equivalents (TE)/g, followed by that of 62.6 μmol of TE/g for 2L1 and 60.6 μmol of TE/g for 2L2, respectively (**Figure 3.1**). Most diploid leaf samples had higher RDSC values than the tetraploid leaf samples, except 2L4. The diploid whole-plant samples generally had higher RDSC values than the tetraploid whole-plant samples except 4W2. The leaf samples, regardless of genotypes, showed higher RDSC than the whole-plant samples. The RDSC values ranging from 18.6 to 68.6 μmol of TE/g for the 16 GP samples were comparable to that of 10.8-67.7 μmol of TE/g for the five commercial GP samples with ethanol as the extraction solvent (Xie et al., 2010). The RDSC showed a good correlation ($R = 0.989$, $P < 0.0001$) with TPC and an inferior correlation ($R = 0.918$, $P < 0.0001$) with TFC, inferring that phenolic compounds might play an important role in the overall DPPH radical scavenging capacity of GPs. Also found was the correlation between RDSC and the rutin and quercetin content ($R = 0.744$, $P < 0.001$), suggesting these individual flavonoid content might be related to the DPPH radical scavenging capacity.

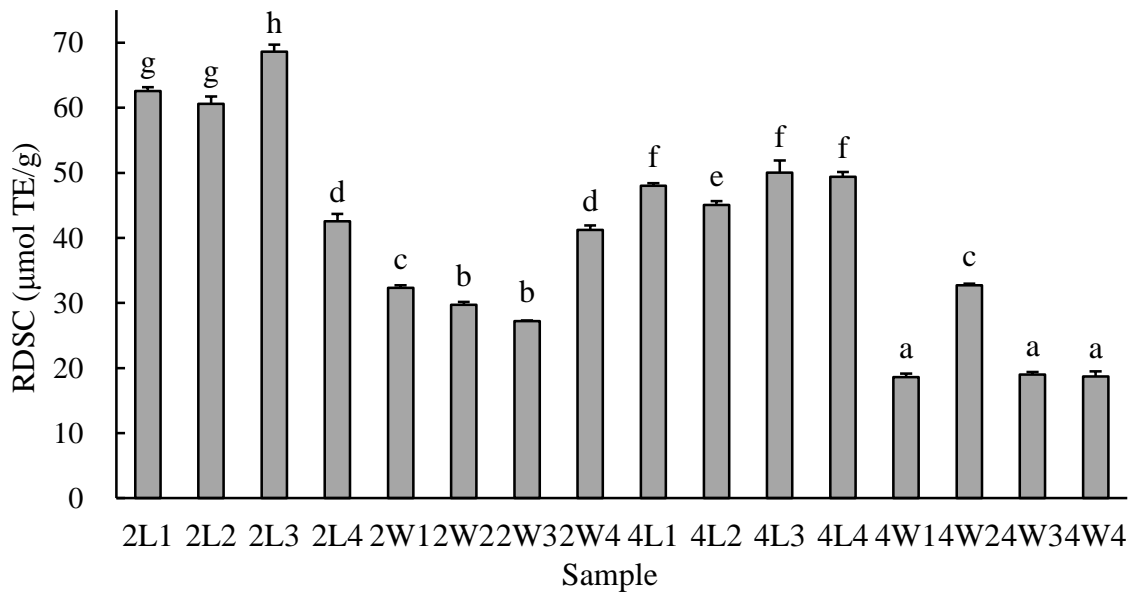


Figure 3.1. Relative DPPH Radical Scavenging Capacity (RDSC) of *G. pentaphyllum* Extracts.

2L1-2L4 represent four diploid leaf samples; 2W1-2W4 represent four diploid whole-plant (leaf and stem) samples; 4L1-4L4 represent four tetraploid leaf samples and 4W1-4W4 represent four tetraploid whole-plant (leaf and stem) samples. TE stands for the trolox equivalents. Data are on an 'as-it-is' botanical weight basis. The vertical bars represent the standard deviation ($n = 3$) of each data point. Different letters represent significant differences ($P < 0.05$).

3.4.5 Hydroxyl Radical Scavenging Capacity (HOSC)

Figure 3.2 shows hydroxyl radical scavenging capacity for the ethanol extracts of the sixteen *G. pentaphyllum* samples. To avoid the solvent interference in the assay, ethanol was replaced by 50% acetone prior to the determination. The highest HOSC of 666.8 μmol of TE/g botanical was obtained for the diploid leaf sample 2L3, and the lowest HOSC was observed in the three tetraploid whole plant GP samples, 4W1, 4W3 and 4W4. The leaf samples had greater HOSC than the whole-plant samples of the same genotype, and the diploid whole-plant samples had stronger DPPH radical scavenging activities than their counterpart tetraploid samples (**Figure 3.1**). The HOSC values of the sixteen GP samples were 134.0-666.8 μmol of TE/g, comparable to that of 82-447 μmol of TE/g observed for the five commercial GP samples using ethanol as the extraction solvent in a recent study (Xie et al., 2010). Pearson correlation test showed that HOSC values were correlated to total phenolic content ($R = 0.913$, $P < 0.001$), total flavonoid content ($R = 0.879$, $P < 0.001$), total rutin and quercetin content ($R = 0.558$, $P < 0.025$) and DPPH radical scavenging capacity ($R = 0.921$, $P < 0.001$). These data suggested that phenolics and flavonoids might be the primary component contributing to the observed antioxidant capacity.

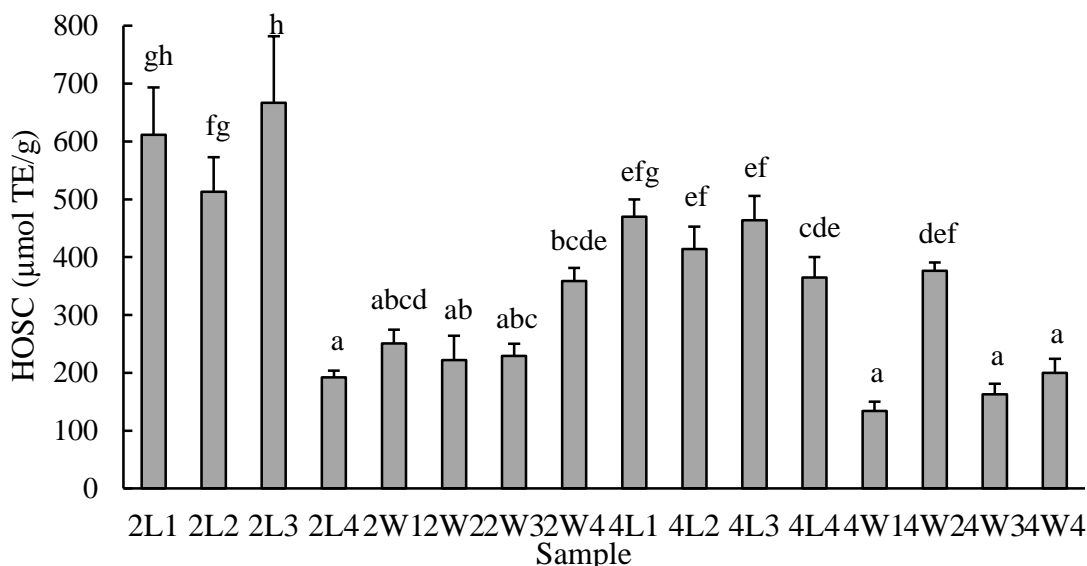


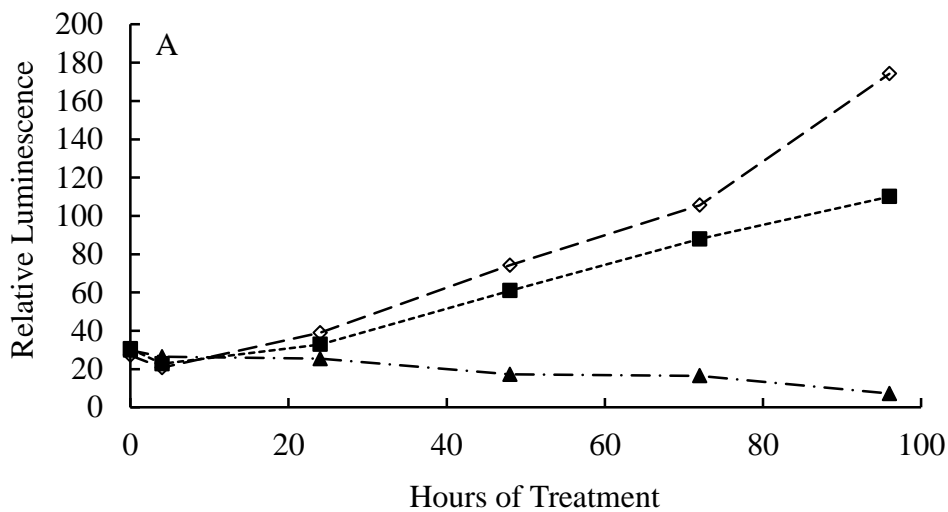
Figure 3.2. Hydroxyl Radical Scavenging Capacity (HOSC) of *G. pentaphyllum* Extracts.

2L1-2L4 represent four diploid leaf samples; 2W1-2W4 represent four diploid whole-plant (leaf and stem) samples; 4L1-4L4 represent four tetraploid leaf samples and 4W1-4W4 represent four tetraploid whole-plant (leaf and stem) samples. TE stands for the trolox equivalents. Data are based on an ‘as-it-is’ botanical weight basis. The vertical bars represent the standard deviation ($n = 3$) of each data point. Different letters represent significant differences ($P < 0.05$).

3.4.6 Effects of *G. pentaphyllum* Components on HT-29 Cell Proliferation

Numerous studies have indicated that *G. pentaphyllum* may be able to inhibit the invasion and migration of cancer cells and induce G0/G1 cell cycle arrest (Chen et al., 2009a; Lu et al., 2008a). In this study, the potential anti-proliferative effect of GP samples was investigated. Anti-proliferative effects against HT-29 colon cancer cells were shown at concentrations of 1 and 10 mg GP botanical equivalents (BE)/mL of culture media (**Figure 3.3**). All samples showed dose- and time-dependent inhibitory activity against the cell growth (**Figure 3.3A**). At the concentration of 1 mg BE/mL of media, the diploid leaf sample extract (2L) had the strongest inhibitory activity. The inhibition rate of 2L extract at a final concentration of 1 mg BE/mL was approximately 73% after 96 hours of treatment. The diploid whole-plant sample (2W)

and the tetraploid leaf sample (4L) resulted in 30-40% inhibition of cell growth, whereas the tetraploid whole-plant sample (4W) had least cell growth suppression capacity. At 10 mg BE/mL concentration, 2L, 2W and 4L extracts had almost 100% inhibition after 96 h treatment, and 4W treatment had about 80% inhibition under the same conditions. The diploid GP might have stronger antiproliferative effect in HT-29 cancer cells than tetraploid counterpart on per botanical weight concentration basis, and the leaf might contain higher content of anti-proliferative components than the whole-plant GP regardless of genotype (**Figure 3.3B**). Samples with higher TPC and TFC trended to have stronger antiproliferative activity against HT-29 cancer cells, although the correlation tests could not be conducted due to the limited sample size. Additional research is required to identify the individual anti-proliferative components in the 100% ethanol extracts of *G. pentaphyllum*.



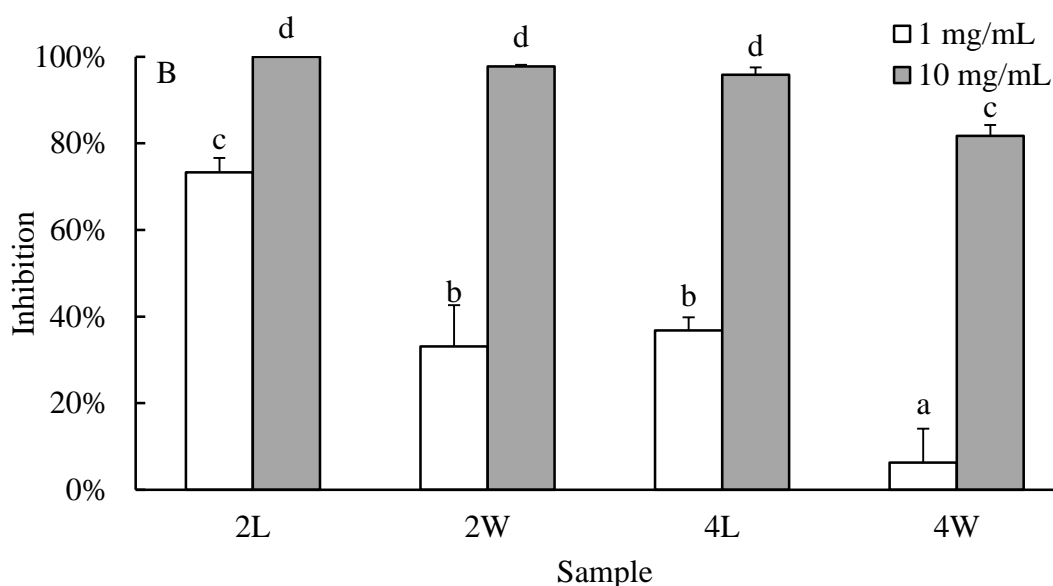


Figure 3.3. Effects of *G. pentaphyllum* Extracts on HT-29 Colon Cancer Cell Growth.

A) represents dose- and time-dependent effect of GP samples (4L). The final concentrations of extracts were 0 (◇), 1 (■), and 10 (▲) mg of botanical equiv/mL in the initial culture media. B) represents inhibitory rate of diploid leaf samples (2L), diploid whole-plant (leaf and stem) samples (2W), tetraploid leaf samples (4L), and tetraploid whole-plant (leaf and stem) samples (4W) after 96 h treatment time. The vertical bars represent the standard deviation ($n = 3$) of each data point. Different letters represent significant differences ($P < 0.05$).

3.4.7 Effects of *G. pentaphyllum* Components on LNCaP Cell Proliferation

The antiproliferative effect of *G. pentaphyllum* ethanol extracts was also investigated using LNCaP prostate cancer cells. **Figure 3.4** shows the inhibition rate of the four GP samples including diploid leaf and whole plant, and tetraploid leaf and whole plant samples at different concentrations after 96 h treatments. The four GP extracts significantly differed in their antiproliferative properties against LNCaP cells, although they all dose-dependently suppressed LNCaP cell growth (**Figure 3.4**). The tetraploid whole-plant sample (4W) did not show any inhibition at 20 μ g BE/mL concentration. At a concentration of 100 μ g BE/mL of media, 4L had strongest

inhibitory activity, followed by the tetraploid leaf sample 4W. When exposed to a dosage level of 200 µg BE/mL, diploid leaf sample 2L showed the strongest inhibitory activity against LNCaP cells, followed by 4L and 2W.

It is widely accepted that individual chemical components may have different antiproliferative mechanisms against cancer cells thus may have different inhibitory manners in different cancer cells (Casagrande & Darbon, 2001; Kampa, Alexaki, Notas, Nifli, Nistikaki, Hatzoglou et al., 2004). In other words, a selected cancer cell line might compensate for low-level toxicity of a bioactive compound but could be significantly affected by the same compound at a higher dose (Zbasnik, Carr, Weller, Hwang, Wang, Cuppett et al., 2009). It is also well known that individual cancer cell lines may respond to a selected bioactive component differently, which is important for discovery of selective inhibitors with fewer side effects against each type of cancer cells. In the present study, HT-29 colon and LNCaP prostate cancer cell lines were included to better understand the potential antiproliferative effects of the four GP samples. Taking together the antiproliferative data on both cancer cell lines, GP components may suppress both HT-29 colon and LNCaP prostate cancer cell proliferation and may be more effective in inhibiting LNCaP prostate cancer cell proliferation on a per botanical weight basis, suggesting the possible selective inhibition of these GP components. It is also possible that the leaf and whole-plant samples of di-and tetraploid GP may differ in their compositions of anti-proliferative components. Since colon and rectal cancer is the second leading cause of cancer death, and prostate cancer is the fifth most common cause of cancer death in the United States (National Cancer Institute, <http://www.cancer.gov>), the potential anti-

proliferative activities may promote the future use of *G. pentaphyllum* in reducing the risk of cancers.

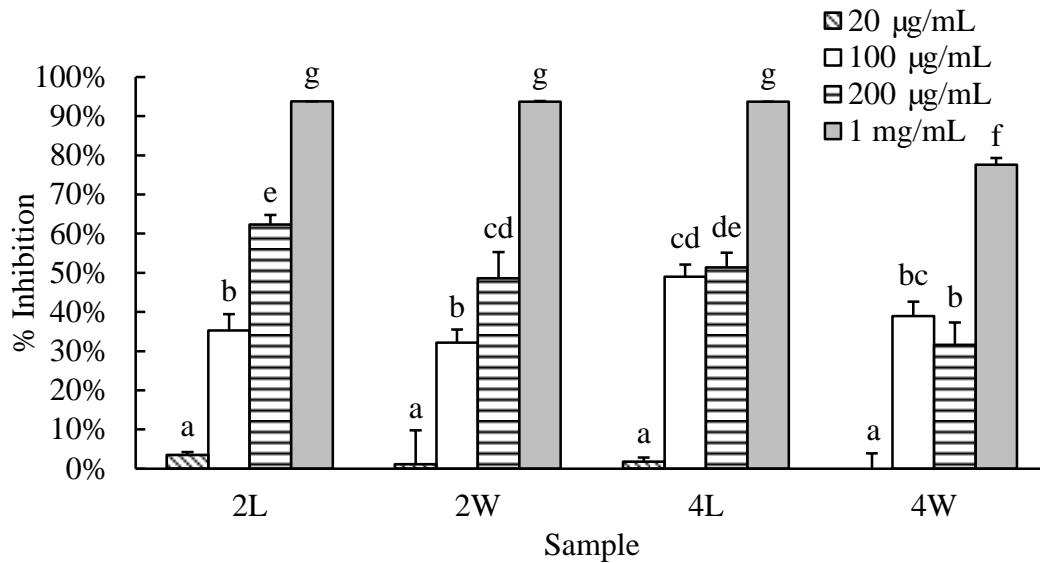


Figure 3.4. Effects of *G. pentaphyllum* Extracts on LNCaP Prostate Cancer Cell Growth.

2L, 2W, 4L and 4W represent diploid leaf samples, diploid whole-plant samples (leaf and stem), tetraploid leaf samples, and tetraploid whole-plant (leaf and stem) samples of *G. pentaphyllum*, respectively. The inhibitory rate was obtained after 96 h treatment time. The vertical bars represent the standard deviation ($n = 3$) of each data point. Different letters represent significant differences ($P < 0.05$).

3.4.8 Effects of *G. pentaphyllum* Extracts on TNF- α , IL-6 and Ptgs2 mRNA expression in LPS-stimulated Mouse J774A.1 Macrophage Cells

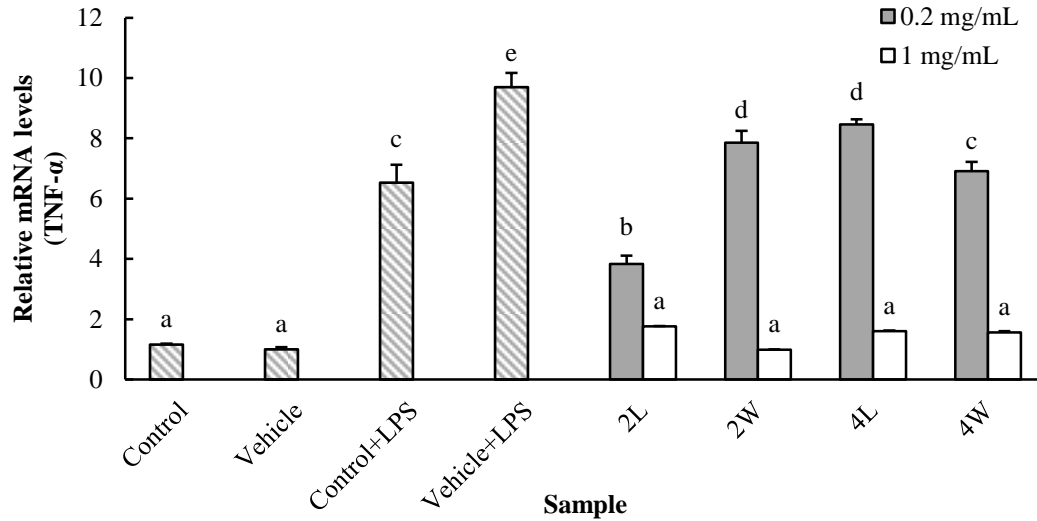


Figure 3.5. Effects of *G. pentaphyllum* on TNF- α mRNA Expressions in Mouse J774A.1 Macrophage Cells.

2L, 2W, 4L and 4W represent diploid leaf samples, diploid whole-plant samples (leaf and stem), tetraploid leaf samples, and tetraploid whole-plant (leaf and stem) samples of *G. pentaphyllum*, respectively. The final concentrations of extracts were 0.2 and 1 mg of botanical equiv/mL in the initial culture media. The vertical bars represent the standard deviation ($n = 3$) of each data point. Different letters represent significant differences ($P < 0.05$).

The anti-inflammatory effect of GP extracts were determined by measuring the mRNA expression in mouse J774.1 macrophage cells stimulated by lipopolysaccharide. As shown in **Figure 3.5**, all four samples significantly suppressed the LPS-induced TNF- α mRNA expression at an initial concentration of 0.2 mg botanical equivalents (BE)/mL media. The diploid leaf sample (2L) showed strongest suppression, followed by tetraploid whole-plant sample (4W). The suppression of LPS-induced TNF- α mRNA expression was dose dependent (**Figure 3.5**). At a higher treatment concentration of 1 mg BE/mL, all four GP samples completely inhibited the expression of TNF- α mRNA, which were not significantly different to that of the vehicle and the control cells.

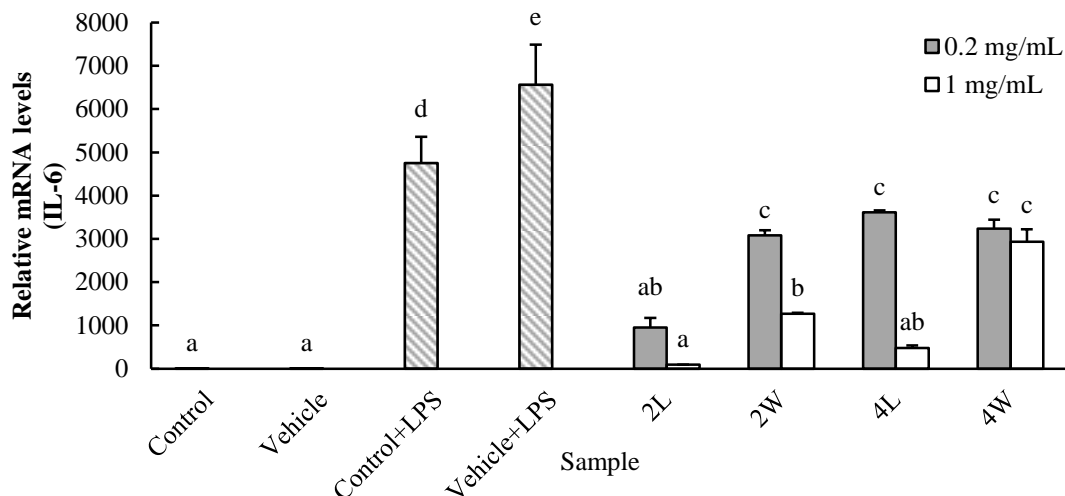


Figure 3.6. Effects of *G. pentaphyllum* on IL-6 mRNA Expressions in Mouse J774A.1 Macrophage Cells.

2L, 2W, 4L and 4W represent diploid leaf samples, diploid whole-plant samples (leaf and stem), tetraploid leaf samples, and tetraploid whole-plant (leaf and stem) samples of *G. pentaphyllum*, respectively. The final concentrations of extracts were 0.2 and 1 mg of botanical equiv/mL in the initial culture media. The vertical bars represent the standard deviation ($n = 3$) of each data point. Different letters represent significant differences ($P < 0.05$).

The IL-6 mRNA expression was inhibited in the presence of 0.2 mg BE/mL GP extracts (**Figure 3.6**). The diploid leaf extract 2L had strongest inhibition on the IL-6 mRNA expression, whereas 4W showed weakest inhibition at the initial concentration of 1 mg BE/mL media. The inhibition was dose dependent (**Figure 3.6**). Diploid GP samples had same or stronger suppressing effect than their tetraploid counterpart samples, and leaf samples showed same or greater inhibitory activities on IL-6 mRNA expression than the whole plant GP samples from the same genotype.

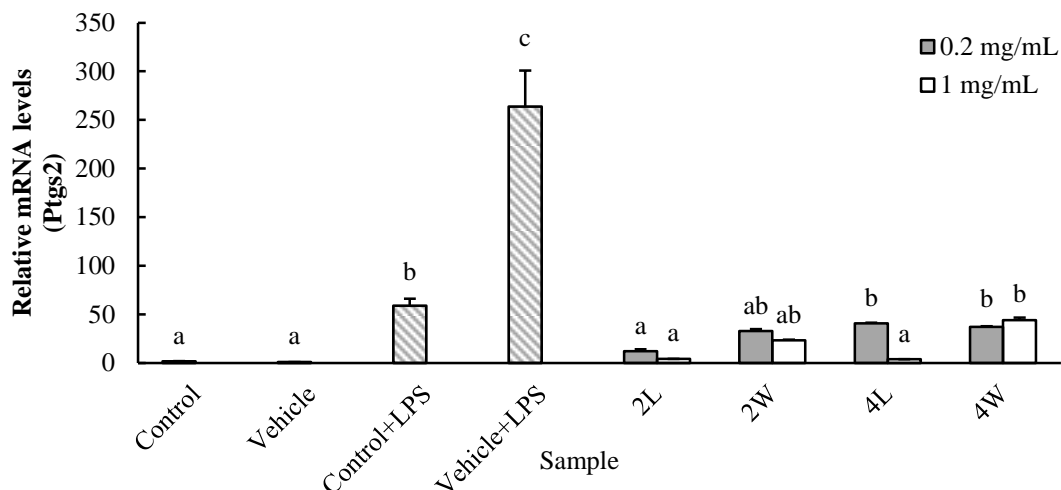


Figure 3.7. Effects of *G. pentaphyllum* on Ptgs2 mRNA Expressions in Mouse J774A.1 Macrophage Cells.

2L, 2W, 4L and 4W represent diploid leaf samples, diploid whole-plant samples (leaf and stem), tetraploid leaf samples, and tetraploid whole-plant (leaf and stem) samples of *G. pentaphyllum*, respectively. The final concentrations of extracts were 0.2 and 1 mg of botanical equiv/mL in the initial culture media. The vertical bars represent the standard deviation ($n = 3$) of each data point. Different letters represent significant differences ($P < 0.05$).

All four GP samples showed strong inhibitory activities on Ptgs2 mRNA expressions in the LPS-stimulated mouse J774A.1 macrophage cells at both 0.2 and 1 mg BE/mL of media (**Figure 3.7**). Diploid leaf (2L) and whole plant (2W) extracts could completely suppress LPS stimulated Ptgs2 mRNA expression at 0.2 mg BE/mL, and the tetraploid leaf GP extract (4L) showed 100% inhibition of the Ptgs2 mRNA expression at 1 mg BE/mL, suggesting that diploid GP might contain higher concentration of the inhibitors than tetraploids. The 4L extract had significantly greater inhibitory effect on Ptgs2 mRNA expression than the 4W extract at 1 mg BE/mL, suggesting that the inhibitors might be more concentrated in the leaf (**Figure 3.7**). Taking together, these GP extracts showed inhibitory effects on expressions of

IL-6, TNF- α , and Ptgs2 mRNAs, suggesting their potential as anti-inflammatory ingredients for nutraceutical and functional food development.

3.4.9 Fiber Content, Selenium Concentration and Moisture Content

Table 3.2. Soluble and Insoluble Fiber Content and Selenium Concentration of *G. pentaphyllum**

	soluble fiber content (g/100 g)	insoluble fiber content (g/100 g)
2L	5.64	20.08
2W	3.94	51.01
4L	4.25	17.09
4W	3.27	46.89

*2L, 2W, 4L and 4W represent diploid leaf sample, diploid whole-plant sample, tetraploid leaf sample, and tetraploid whole-plant samples respectively. Data are on an ‘as-it-is’ botanical weight basis.

Dietary fiber has been widely accepted as a group of beneficial components for reducing the risk of human chronic diseases, and soluble and insoluble fiber preparations may differ in their health effects. Soluble and insoluble fiber contents were determined in the four combined samples. As shown in **Table 3.2**, the diploid leaf sample (2L) had the highest soluble fiber content of 5.6 g/100 g of botanical. The tetraploid leaf sample (2W) had the second highest soluble fiber content of 4.3 g/100 g. The highest insoluble fiber content (51.0 g/100 g) was found in the diploid whole-plant sample, which was followed by tetraploid whole-plant sample (46.9 g/100 g). It could be concluded that the diploid samples had greater fiber content than their counterpart tetraploid samples, and the leaf sample with the same genotype had more soluble fiber and the whole-plant sample of the same genotype had more insoluble fiber.

Selenium is an important micronutrient that attracts much attention recently. Selenium concentrations were determined in all sixteen GP samples, which were grown in Se-rich soil. The highest Se content of 6.47 mg/g was detected in the tetraploid leaf sample 4L4 under the experimental conditions. As shown in **Table 3.1**, the tetraploid samples may have accumulated more Se content than the diploid samples in general. The moisture content of the sixteen GP samples ranged from 0.57% to 5.65%. The highest water content was detected in diploid leaf sample (2L2), while the lowest was detected in tetraploid whole-plant sample (4W4).

In summary, this study demonstrated that different genotypes (diploid vs tetraploid) and different parts (leaf vs whole-plant) of *G. pentaphyllum* might differ in health components and properties. The results from this study also suggested the potential effect of growing environment and the interaction between genotype and growing environment may alter the compositions of beneficial components and nutraceutical properties of GP. Further investigation is required to understand the accurate differences of bioactive components among *G. pentaphyllum* samples, the factors altering the GP health properties, and the mechanism involved in their beneficial activities.

Chapter 4: Chromatographic fingerprint analysis, and rutin and quercetin compositions in the leaf and whole-plant samples of diploid and tetraploid *Gynostemma pentaphyllum*

Xie, Z.; Zhao, Y.; Chen, P.; Jing, P.; Yue, J.; Yu, L. (2011). *J. Agric. Food Chem.* 59, 3042-3049

4.1 Abstract

Gynostemma pentaphyllum (Thunb.) Makino, also known as jiaogulan, has been shown to have antioxidant, antiproliferative, and anti-inflammatory activities. Flavonoid is considered a major contributor for these beneficial effects. To obtain chemical patterns of flavonoids in *G. pentaphyllum* of different genotypes (di- versus tetraploids) and different parts (leaf versus whole-plant) of plants, the extraction condition was optimized and a fingerprinting approach was established by means of high-performance liquid chromatography coupled with diode array detection and mass spectrometry (HPLC-DAD-MS). Eight flavonoids were identified, among which rutin and quercetin were quantified. The highest levels of rutin and quercetin were 23.03 and 12.10 mg/g, respectively, observed in the diploid leaf sample 2L3 and 2L2, while the lowest levels of rutin and quercetin were 1.92 and 0.25 mg/g in the tetraploid whole-plant sample 4W3. The chemical patterns were further analyzed by similarity calculation and principal component analysis (PCA). Seven common characteristic peaks were found in all of the tested samples. Flavonoid patterns of tetraploids were significantly different from those of diploids, whereas different parts of plants showed less difference. The flavonoid pattern of the diploid leaf sample was most similar to that of the reference botanical *G. pentaphyllum*. The combination of

chromatographic fingerprint and quantification analysis could be used for quality assessment of *G. pentaphyllum* and its derived nutraceutical products.

4.2 Introduction

Gynostemma pentaphyllum Makino, known as Jiaogulan, is a perennial liana plant, and has been traditionally used in food, tea, and folk medicines in many East and Southeast Asian countries for its possible health properties such as reducing the risk of cardiovascular diseases (Tanner et al., 1999; Wang et al., 2007), and hypoglycemic (Yeo et al., 2008), anti-inflammatory (Lin et al., 1993; Xie et al., 2010b), and anticancer (Lu et al., 2008b) activities. Our recent study showed that commercial *Gynostemma pentaphyllum* Makino products contained significant levels of natural antioxidants, and might suppress IL-6, Ptg2 and TNF- α mRNA expression and inhibit HT-29 human cancer cell proliferation (Xie et al., 2010b). Flavonoids were considered one of the major components that contribute to the health beneficial properties of *G. pentaphyllum* (Kao et al., 2008). Several flavonoids have been identified and quantified in a *G. pentaphyllum* sample (Kao et al., 2008). These included quercetin-di-(rhamno)-hexoside (548.3 $\mu\text{g/g}$), quercetin-rhamno-hexoside (1249.6 $\mu\text{g/g}$), kaempferol-rhamno-hexoside (1792.9 $\mu\text{g/g}$), kaempferol-rhamno-hexoside (2416.5 $\mu\text{g/g}$), rutin (1602.8 $\mu\text{g/g}$), kaempferol-rhamno-hexoside (170.7 $\mu\text{g/g}$) and kaempferol-3-O-rutinoside (429.7 $\mu\text{g/g}$) (Kao et al., 2008). In addition, a recent study from our laboratory showed that five different commercial *G. pentaphyllum* samples significantly differed in their total flavonoid, phenolic and saponin contents, and their rutin and quercetin concentrations (Xie et al., 2010b). The five *G. pentaphyllum* samples also differed in their radical scavenging capacities,

ability to suppress IL-6, Ptg2 and TNF- α mRNA expressions, and antiproliferative effects on HT-29 human colon cancer cell lines (Xie et al., 2010b). These findings suggested the potential variation in the chemical compositions and health properties of *G. pentaphyllum* samples from different genotypes harvested in different seasons at different locations.

As a continuation of our research on *Gynostemma pentaphyllum* Makino, the present study compared the diploid and tetraploid *G. pentaphyllum*, and their leaf and whole-plant samples for their rutin and quercetin contents, and their high-performance liquid chromatography-mass spectrometry (HPLC-MS) fingerprints of flavonoids analyzed by similarity calculation and principal component analysis (PCA). A standard *G. pentaphyllum* botanical sample was included as a reference for HPLC fingerprinting study, and four diploid leaf (2L1-2L4), four diploid whole-plant (2W1-2W4), four tetraploid leaf (4L1-4L4) and four tetraploid whole-plant (4W1-4W4) samples from the same growing location were involved to test how the diploid and tetraploid samples, and how leaf and the whole-botanical samples may differ to each other in their flavonoids profiles. To the best of our knowledge, this is the first time flavonoid fingerprint of *G. pentaphyllum* was investigated, and this is also the first report of tetraploid *G. pentaphyllum* samples for their rutin and quercetin contents, and their flavonoid fingerprint. The results from this study will be used to assess and improve the quality of *Gynostemma pentaphyllum* and to promote their use in functional foods to improve human health.

4.3 Materials and Methods

4.3.1 Chemicals and Materials

Pure rutin and quercetin were purchased from Extrasynthese (Genay, Cedex, France) and their purities were above 98% by HPLC analysis. HPLC-grade acetonitrile was purchased from VWR International, Inc. (Clarksburg, MD). HPLC-grade water was prepared from distilled water using a Milli-Q system (Millipore Laboratory, Bedford, MA). HPLC-grade formic acid was purchased from Fluka Analytical (Buchs, Switzerland). All other chemicals and solvents were of the highest commercial grade and used without further purification. Sixteen *Gynostemma pentaphyllum* Makino samples were obtained from the Asian Citrus Holdings Limited (Hong Kong), including four diploid leaf samples (2L1-2L4), four diploid whole-plant (stems and leaves) samples (2W1-2W4), four tetraploid leaf samples (4L1-4L4), and four tetraploid whole-plant (stems and leaves) samples (4W1-4W4). A *Gynostemma pentaphyllum* Makino reference botanical was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). All botanical samples were grown in the Dabashan area of Shaanxi province of China in 2009, and harvested by hands from the different locations of the same field according to a standard randomization protocol. The samples were washed using tap water, dried in a tea drier at 70-76 °C, pulverized with a conventional pulverizing machine, and kept in zip lock bags in dark until analysis. The ploidy was determined by root tip chromosome checking (22 vs. 44).

4.3.2 Sample and Reference Preparation

The reference chemicals, i. e. rutin and quercetin were dissolved in methanol at 0.1432 and 0.1076 mg/mL as stock solution, respectively. The concentration range used for quantification was 0.001432-0.1432 mg/mL for rutin and 0.001076-0.1076 mg/mL for quercetin.

For sonication extraction, 0.5 g pulverized samples were accurately weighed and extracted with 10 mL methanol, ethanol, methanol:water (1:1, v/v), methanol:chloroform (1:1, v/v) and acetone, respectively, using sonication with an FS30 Ultrasonic sonicator (40 kHz, 100 W) (Fisher Scientific, Pittsburgh, PA) for 1 h at room temperature. The mixtures were centrifuged at 5000 rpm for 5 min and filtered through a 0.45 µm membrane filter before further investigation.

For Soxhlet extraction, 5 g pulverized samples were accurately weighed and extracted in pure methanol for 6 h using a Soxhlet apparatus. The sample solutions were filtered through a 0.45 µm membrane filter before further investigation.

4.3.3 Total Flavonoid Content

Total flavonoid content was determined according to an aluminum colorimetric method described previously (Quettier-Deleu et al., 2000). Briefly, the sample extract/standard (150 µL) was mixed with 1 mL of 5% sodium nitrite for 6 min, followed by adding 1 mL of 10% aluminum nitrate and 4 mL of 4% sodium hydroxide. Rutin was used as the standard with a range from 0.812 to 4.06 mg/mL. Absorbance was read at 502 nm on a Genesys 20 spectrophotometer (Thermo

Scientific, Waltham, MA) after 15 min of reaction. The results were reported as mg rutin equivalents per g of botanicals.

4.3.4 HPLC-DAD-MS Procedure

The fingerprint was determined by HPLC-DAD analysis according to the protocol described previously with modification (Chen et al., 2007). The tests were carried out by Agilent Technologies (Palo Alto, CA, USA) 1100 Series system comprising a binary pump with a vacuum degasser, a thermostatted column compartment, an autosampler, and a diode-array detector (DAD). Compounds were separated on a reversed phase column (Agilent Eclipse Plus C18, 150 mm × 2.1 mm, 1.8 µm) at a flow rate of 0.25 mL/min. The HPLC separation was accomplished using a two-solvent gradient system. The mobile phases consisted of water (containing 0.1% formic acid, A) and acetonitrile (containing 0.1% formic acid, B). The initial ratio of A to B was 90:10 (v/v); this was changed linearly to 55:45 in 20 min, to 10:90 after 30 min, and then kept at 10:90 from 30 min to 40 min. The wavelength range was 190-400 nm for detection of flavonoids and chemical fingerprint, and 256 nm for quantitative analysis. The column temperature was set at 45 °C. The compounds were identified by HPLC-MS. The tests were performed by Finnigan LCQ Classic ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) using an ESI interface. The conditions of MS analysis were as follows: sheath gas, 80 arb; auxiliary gas, 10 arb; spray voltage, 4.5 kV; capillary temperature, 220 °C; scan mode: positive and negative; capillary voltage: 18/-12 V; MS full scan range, m/z 100-1500; collision energy for collision induced dissociation (CID), 25-50%; source fragmentation voltage: 20%; isolation width, 3.0 Th.

4.3.5 Data Analysis

Data from total flavonoid, rutin, and quercetin content were reported as mean \pm SD for triplicate determinations on an ‘as-it-is’ botanical weight basis. One-way ANOVA and Tukey’s test were employed to identify differences in means.

The similarity was calculated based on the information obtained from entire chromatographic profiles. Data analysis was performed by a software named Similarity Evaluation System (SES) for Chromatographic Fingerprint of Traditional Chinese Medicine Ver. 2004A (Chinese Pharmacopoeia Commission, China), which was recommended by the State Food and Drug Administration of China (SFDA). The correlation coefficients of entire chromatographic profiles of samples were calculated.

Principal component analysis (PCA) was carried out using software of SIMCA-P + 11.5 (Umetrics AB, Sweden) based on UV (256 nm) data.

4.4 Results and Discussion

4.4.1 Selection of the Extraction Condition

Flavonoids have been considered as one of the major health-beneficial components in *G. pentaphyllum* botanicals. Modified from recent studies, several solvent systems including methanol, ethanol, methanol:water (1:1, v/v), methanol:chloroform (1:1, v/v) and acetone with sonication or Soxhlet procedures were evaluated and compared for *G. pentaphyllum* flavonoid extraction (Kao et al., 2008; Lin et al., 2000; Lin & Harnly, 2010; Tsai et al., 2010a). As shown in **Table 4.1**, Soxhlet extraction with methanol as the solvent for 6 hours was most effective among

the tested protocols, and obtained the highest total flavonoid content of over 45 mg rutin equivalents per gram of botanicals, along with the greatest rutin and quercetin concentrations of 11 mg/g and 1 mg/g on a per botanical weight basis. These data suggested that Soxhlet extraction with methanol is a preferred method for estimating the *G. pentaphyllum* flavonoids in the botanicals. Also noted was that methanol with sonication obtained the second highest total flavonoids and the highest rutin content, and could be a method for comparing a group of *G. pentaphyllum* botanical samples for their flavonoids contents and compositions (Table 4.1). MeOH-H₂O with higher polarity than MeOH did not significantly enhance the extraction of rutin nor reduce quercetin extraction (Table 4.1). However, replacing MeOH-H₂O with other solvents such as DMSO for different measurements such as cell proliferation or antioxidant activities will involve lyophilization, therefore will be much harder than using MeOH as solvent, suggesting that MeOH-H₂O is not a preferred solvent for extraction of *G. pentaphyllum* flavonoids.

Table 4.1. Effects of Extraction Solvent and Method on Phytochemical Properties of *G. pentaphyllum**

	Solvent	TFC (mg RE/g)	Rutin content (mg/g)	Quercetin content (mg/g)
Sonication	MeOH	37.54 ± 1.86d	9.79 ± 0.05d	0.60 ± 0.00c
	EtOH	20.44 ± 1.64b	4.19 ± 0.57c	0.30 ± 0.04b
	MeOH:H ₂ O (1:1, v/v)	30.46 ± 1.00c	10.94 ± 0.85d	0.50 ± 0.06c
	MeOH:CHCl ₃ (1:1, v/v)	28.11 ± 2.39c	2.66 ± 0.15b	0.52 ± 0.01c
	Acetone	12.25 ± 0.51a	1.04 ± 0.04a	0.14 ± 0.00a
Soxhlet	MeOH	45.63 ± 2.82e	11.06 ± 0.02d	1.02 ± 0.01d

* Sample 4L3 was used in all tests. Different letters represent significant differences ($P < 0.05$). TFC stands for total flavonoid content by spectrometric methods. RE stands for rutin equivalents. Rutin and quercetin content were flavonoid profile obtained by HPLC.

4.4.2 Rutin and Quercetin Contents

Table 4.2. Rutin and Quercetin Contents in *G. pentaphyllum**

	Rutin (mg/g)	Quercetin (mg/g)
2L1	18.97 ± 0.06h	9.64 ± 0.02i
2L2	22.98 ± 0.25j	12.10 ± 0.07m
2L3	23.03 ± 0.17j	11.45 ± 0.02l
2L4	20.97 ± 0.29i	11.10 ± 0.06k
2W1	14.15 ± 0.12g	4.64 ± 0.02h
2W2	9.15 ± 0.02d	3.60 ± 0.02g
2W3	8.17 ± 0.02c	1.00 ± 0.03e
2W4	14.13 ± 0.09g	10.14 ± 0.05j
4L1	11.19 ± 0.04f	1.27 ± 0.03f
4L2	9.74 ± 0.06d	0.82 ± 0.04d
4L3	11.07 ± 0.00ef	1.02 ± 0.01e
4L4	10.29 ± 0.03de	0.92 ± 0.01de
4W1	3.90 ± 0.02b	0.52 ± 0.00bc
4W2	6.86 ± 0.04c	0.66 ± 0.01c
4W3	1.92 ± 0.00a	0.25 ± 0.00a
4W4	3.19 ± 0.04b	0.47 ± 0.01b

*2L1-2L4 represent diploid leaf botanicals; 2W1-2W4 represent diploid whole-plant botanicals; 4L1-4L4 represent tetraploid leaf botanicals; 4W1-4W4 represent tetraploid whole-plant botanicals. Different letters represent significant differences ($P < 0.05$).

Flavonoids are a group of polyphenolic phytochemicals, which may be the major contributors for the health properties of vegetables, fruits (grapes, cranberries) and other botanical products (Parry et al., 2006). Differences in type and amount of flavonoids exist in botanicals of different varieties and tend to follow taxonomic lines. Therefore it's proposed that *G. pentaphyllum* of different genotypes may have different chemical compositions on flavonoid profiles. Rutin and quercetin are two of the most abundant flavonoids found in *G. pentaphyllum* (Kao et al., 2008; Xie et al., 2010b). For the first time, the present study determined the rutin and quercetin contents in tetraploid *G. pentaphyllum* botanical samples. The leaf samples of tetraploid had a rutin content of 9.7-11.2 mg/g and a quercetin content of 0.8-1.3 mg/g, and the whole-botanical samples of tetraploid had significantly less rutin and

quercetin at levels of 1.9-6.7 and 0.3-0.7 mg/g, respectively (**Table 4.2**). The concentrations are reported on a per 'as it is' weight basis. The rutin contents in these tetraploid samples are similar to that in a recent study (1.6 mg/g) (Kao et al., 2008). The levels of rutin and quercetin are comparable to those of commercial *G. pentaphyllum* products (0.6-11.2 mg/g and 0.1-7.4 mg/g, respectively) in our previous study (Biasioli et al., 2003). It was also observed that the diploid leaf samples had greater rutin and quercetin levels than their counterpart whole-botanical samples (**Table 4.2**). These data indicated that rutin and quercetin and possibly total flavonoids are more concentrated in the leaf samples than that in the stems and other non-leaf parts of the plant.

In addition, the rutin and quercetin contents of the diploid and tetraploid *G. pentaphyllum* were compared. Diploid leaf and whole-botanical samples had significantly greater rutin and quercetin contents than their tetraploid counterparts (**Table 4.2**). Diploid leaf sample 2L3 had the highest rutin concentration of 23.03 mg/g. Rutin contents in the four tested diploid *G. pentaphyllum* samples ranged from 19-23 mg/g, which were 2 times higher than those in the tetraploid leaf samples, respectively. The quercetin contents in the diploid samples were about 10 times of those detected in the tetraploid *G. pentaphyllum*. Rutin and quercetin contents in the diploid whole-botanical samples were also significantly higher than those in the tetraploid samples (**Table 4.2**). These results indicated that the diploid *G. pentaphyllum* may contain greater amount of rutin, quercetin, and total flavonoids than their tetraploid counterparts from the same growing locations.

4.4.3 Validation of Chromatographic Fingerprinting Methodology

The chromatographic fingerprinting methodology was validated for its precision, repeatability and stability. The precision was evaluated by analysis of five injections of the same testing sample solution consecutively. The repeatability was examined by determination of five different working sample solutions prepared from the same botanical sample. The stability was examined by analysis of sample solution at different time points (0, 2, 4, 8, 16, and 24 h). The similarities of all the tests were above 0.900, indicating that the HPLC fingerprint analysis method was valid and effective.

The fingerprinting analysis was performed using the characteristic peak approach. The detailed process has been discussed previously (Chen et al., 2007; Chen, Song, & Lin, 2009c). In this approach, an authentic sample of a certain botanical was selected as a reference fingerprint (RF, in this study, a *G. pentaphyllum* reference from the National Institute for the control of Pharmaceutical and Biological Products). The most obvious peak of the RF was selected as the reference peak (RP, in this study, peak 5, rutin). The areas of all other peaks in the chromatograms were normalized against the area of the RP and the ratios of the peaks were entered into a peak table and used for PCA analysis. As shown in **Figure 4.1** and **Table 4.3**, a total of twenty-four characteristic peaks were detected in all the tested *G. pentaphyllum* samples. Of the 24 peaks, 8 peaks including peak 5, 6, 8, 10, 11, 14, 17 and 18 were detected in all samples and were designated as “common peaks”. Alignment of retention times of all common peaks were analyzed by SpecAlign 2.4 (Jason Wong, Oxford, UK). To achieve higher accuracy on identification of flavonoids, both

positive and negative ion modes were employed. Peak 5 and peak 14 were identified to be rutin and quercetin, respectively, by comparing the UV spectra, retention time and MS fragmentation behaviors with those of the references (Lin, Chen, & Harnly, 2008a) (**Figure 4.1, Table 4.3**). Peak 1, 2, 3, 4, 8, 9 and 17 were tentatively identified as quercetin-di-(rhamno)-hexoside (Kao et al., 2008), kaempferol 3-O-di-p-coumaroylhexoside (Lin et al., 2008a), kaempferol 3-O-di-p-coumaroylhexoside (Lin et al., 2008a), quercetin-rhamno-hexoside (Kao et al., 2008), kaempferol-rhamno-hexoside (Kao et al., 2008), kaempferol-3-O-rutinoside (Lin & Harnly, 2008b) and kaempferol (Lin et al., 2008a), respectively, based on the MS fragmentation behaviors under the experimental conditions and the literatures (**Table 4.3**).

The HPLC fingerprints of diploid and tetraploid, leaf and whole-botanical samples, and the reference botanical were compared (**Figure 4.1**). REF represents the reference botanical spectrum, 2L2, 2W3, 4L3, and 4W2 are the representative HPLC fingerprints of diploid leaf, diploid whole-botanical, tetraploid leaf, and tetraploid whole-botanical. The HPLC peaks could be generally grouped into regions I, II, III, and IV (**Figure 4.1**). In Region I, four peaks 1-4 (quercetin-di-(rhamno)-hexoside, kaempferol 3-O-di-p-coumaroylhexoside, kaempferol 3-O-di-p-coumaroylhexoside and quercetin-rhamno-hexoside) were exclusively detected in tetraploid *G. pentaphyllum* (4L, 4W), regardless leaf or whole-plant sample. Furthermore, peaks 5 (rutin), 6, 7, 8 (kaempferol-rhamno-hexoside), 10 and 11 were common peaks in all samples in the Region II, except peak 7, which was not detectable in the reference botanical. Peak 9 (kaempferol-3-O-rutinoside) was specific for tetraploid *G. pentaphyllum* (**Figure 4.1**). Diploid (2L2 and 2W3) *G. pentaphyllum* also differed

from the tetraploid (4L3 and 4W2) samples in the Region II by having relatively greater ratio of peak 8 and lower ratio of peaks 6 and 7 (**Figure 4.1**). No difference was observed between the leaf and whole-plant botanical samples for either diploid or tetraploid *G. pentaphyllum* in their HPLC fingerprints in the Region II.

In the region III, Peaks 14 (quercetin) and 17 (kaempferol) were common for all five tested *G. pentaphyllum* samples. Peak 12 was only observed in *G. pentaphyllum* diploid and tetraploid leaf samples and the reference botanical, but not found in any whole-botanical samples. Peaks 13 and 19 were only detected in diploid leaf and the reference botanical samples under the experimental conditions. Peak 18 was found in diploid leaf and whole-botanical and the reference samples, whereas no peaks at the same retention time were detected in tetraploid samples. Peaks 15 and 16 were only detected in the reference sample.

In Region IV, peak 22 was only detected in the diploid leaf and reference botanical samples and, peak 24 was observed only in the diploid leaf and whole-botanical and the reference samples but not in any tetraploid samples (**Figure 4.1**). Peak 23 was only seen in the reference botanical (**Figure 4.1**). Also noticed was that the tested *G. pentaphyllum* samples might differ in their relative levels of individual flavonoids compounds. In addition, the diploid leaf had a HPLC fingerprint most similar to that of the reference botanical sample (**Figure 4.1**).

Table 4.3. MS Fragmentation of the Investigated Compounds by HPLC-MS*

Peak No.	RT	UV (nm)	[M-1]-/[M+1]+	NI/PI	Compound
1	9.63	256,352	755.26/756.75		quercetin-di-(rhamno)-hexoside
2	10.48	264,346	739.23/740.66		kaempferol 3-O-di-p-coumaroylhexoside
3	10.69	266,346	739.30/740.73		kaempferol 3-O-di-p-coumaroylhexoside
4	10.99	264,346	609.21/610.77		quercetin-rhamno-hexoside
5	11.21	256,354	609.19/610.91	1218.87 [2M-1]/633.19 [M+Na]+, 464.95 [M+H-Rham], 303.27[M+H-Rham-Glu]	rutin
6	11.71	256,348	609.23/610.79		unknown
7	12.09	264,346	609.25/609.85		unknown
8	12.66	266,348	593.18/-	285.00 [M-H-Rham-Glu]-/617.17[M+Na]+, 503, 287.32 [M+H-Rham-Glu]+	kaempferol-rhamno-hexoside
9	12.86	256,348	593.21/-	284.71[M-H-Rham-Glu]-/617.18[M+Na]+, 503.01, 287.26 [M+H-Rham-Glu]+	kaempferol-3-O-rutinoside
10	13.05	254,356	623.19/-	315.20 [M-H-Rham-Glu]-/647.21[M+Na]+, 533.02, 317.25 [M+H-Rham-Glu]+	unknown
11	13.17	254,352	623.19/-	315.20 [M-H-Rham-Glu]-/647.24[M+Na]+, 533.02, 317.25 [M+H-Rham-Glu]+	unknown
12	15.52	266,346	607.21/-	299.09 [M-H-Rham-Glu]-/631.24[M+Na]+, 517.08, 301.30 [M-H-Rham-Glu]+	unknown
13	16.84	268,308	697.25/699.14	675, 643, 299.17/659.16, 627.16, 603.10	unknown
14	17.39	256,360	301.07/-		quercetin
15	19.04	352	329.06/331.28	301.15/661.19 [2M+Na]+	unknown
16	19.74	266,348	329.10/331.25	313.12/315.30	unknown
17	20.33	266,368	285.23/287.27		kaempferol
18	21.04	370	315.16/317.22		unknown
19	21.16	298,368	315.27/317.22		unknown
20	24.24	270,364	299.15/-	/603.08	unknown
21	24.34	270,362	299.12/-	/603.06	unknown
22	26.8	266,366	299.16/-		unknown
23	26.98	272,362	299.16/301.28		unknown
24	27.27	256,370	299.23/-		unknown
25	30.93	266,362	/315.30		unknown

*RT, NI and PI stand for retention time, negative ion mode and positive ion mode respectively.

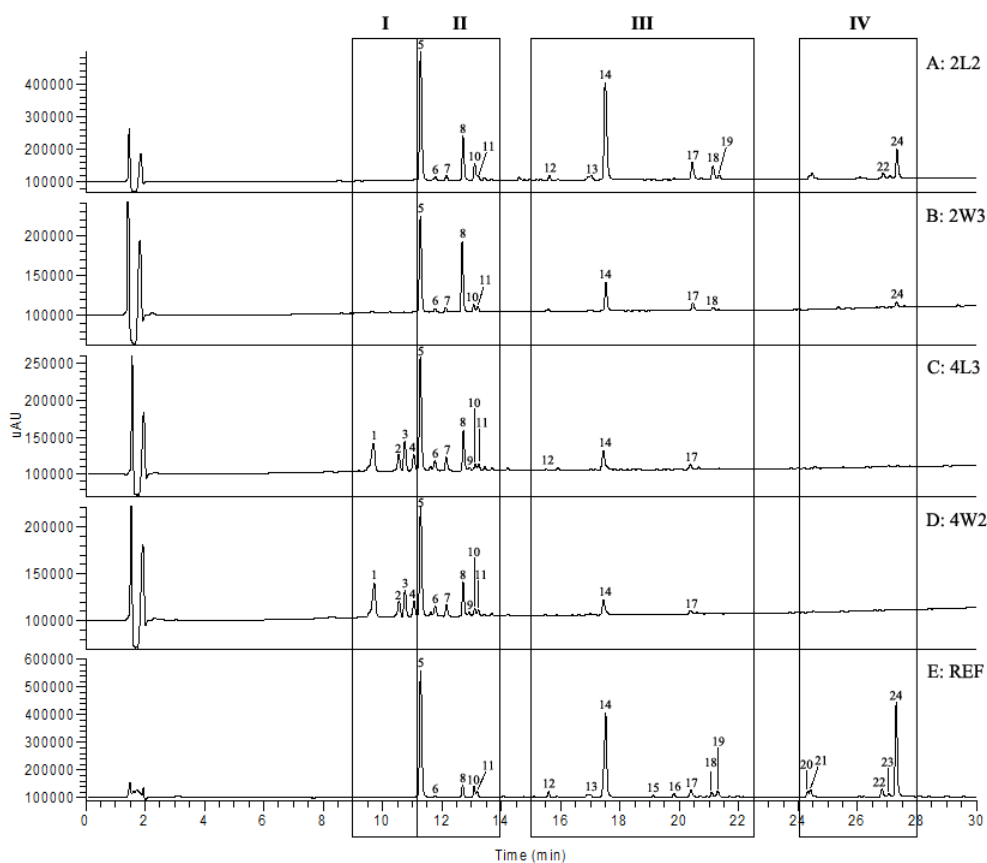


Figure 4.1. HPLC Fingerprint of the Representative *G. pentaphyllum* Samples. A) diploid leaf botanical (2L2); B) diploid whole-plant botanical (2W3); C) tetraploid leaf botanical (4L3); D) tetraploid whole-plant botanical (4W2); E) reference botanical. Data was obtained at 256 nm.

4.4.4 Similarity Calculation of the HPLC Fingerprint Analysis

There are multiple bioactive chemical compounds in herbs and determination of the amounts of one or several “so-called biomarkers” are not enough to evaluate the quality of herbs. Chemical quality control should also consist of chemical fingerprint, which has been introduced and accepted by WHO in 1991 (World Health Organization, 1991) and State Food and Drug Administration (SFDA) of China in 2000 as an efficient approach (State Drug Administration of China, 2000). Chromatographic fingerprint analysis emphasizes on systematic characterization of

chemical composition of the herbal samples. It can give an overview of the chemical pattern of different samples for quality assessment. As a suitable method for quality control, it has been widely studied and applied to different herbs in recent years (Kong, Zhao, Xiao, Jin, & Li, 2009; Xie, Yan, Guo, Lam, Chui, & Yu, 2010a; Ye, Zhang, Dai, Yan, Huang, Liang et al., 2009). Although it is possible to visually differentiate the chromatograms, it will result in subjective and unreliable outcomes. Therefore, the accurate similarity of the chromatograms and the chemical pattern recognition methods should be utilized for fingerprint analysis. The similarity evaluation of fingerprint is one of the most important methods in quality control of TCM (traditional Chinese medicine). Generally, similarity evaluation system (SES) is used for calculation recommended by SFDA. The chromatograms of the samples were exported from LC as AIA or CSV formats and then imported to SES, which were recognized as data points. After the alignment and deconvolution of the original data, mean values or medians were analyzed as correlation coefficients. The correlation coefficients of chromatograms reflect the similarity.

In the present study, SES software was employed to synchronize and quantitatively comparing the tested *G. pentaphyllum* samples, as well as to provide the correlation coefficients among them. The results are shown in **Table 4.4**. The closer the correlation coefficient values were to 1, the more similar the two chromatograms were. The similarity values for samples within the same category, were all above 0.97 except that 2W3 and 2W4 were lower than 0.93 compared to each other and to the other two diploid whole-botanical samples. These data indicated that the entire HPLC flavonoids chromatograms of the *G. pentaphyllum* samples from

the leaf or whole-botanical samples of either diploid or tetraploid genotype were generally consistent and stable.

The similarity values for *G. pentaphyllum* samples 2L1~2L4 to the reference botanical ranged from 0.914286 to 0.936237, and that for 2W4 was 0.933337 (**Table 4.4**), indicating that the flavonoid profiles of 2L1~2L4 and 2W4 were more similar to the reference botanical. Comparing to the reference, the similarity values were below 0.81, 0.49, and 0.47 for *G. pentaphyllum* samples 2W1-2W3, 4L1-4L4, and 4W1-4W4 (**Table 4.4**). Taken together, the similarity analysis data suggested that the diploid leaf *G. pentaphyllum* samples had a flavonoid profile most similar to that of the reference botanical for *G. pentaphyllum*.

Table 4.4. The Similarities of the Different *G. pentaphyllum* Samples

	2L1	2L2	2L3	2L4	2W1	2W2	2W3	2W4	4L1	4L2	4L3	4L4	4W1	4W2	4W3	4W4	REF
2L1	1	0.986124	0.98182	0.982549	0.803492	0.859403	0.656079	0.975242	0.571191	0.496461	0.566273	0.524028	0.48173	0.48772	0.450392	0.481475	0.936237
2L2		1	0.995359	0.995399	0.803781	0.856781	0.654775	0.980101	0.572706	0.492469	0.564566	0.525849	0.472899	0.480477	0.439611	0.470504	0.917554
2L3			1	0.998905	0.812297	0.862195	0.666642	0.981505	0.582484	0.503795	0.573861	0.533374	0.483229	0.490449	0.446903	0.482871	0.920604
2L4				1	0.808627	0.861312	0.663882	0.981636	0.581443	0.500737	0.573	0.533058	0.478852	0.487625	0.443626	0.476198	0.914286
2W1					1	0.982408	0.92577	0.794934	0.821243	0.826733	0.83434	0.793456	0.842474	0.829448	0.829372	0.843837	0.77219
2W2						1	0.915217	0.845239	0.790085	0.780627	0.803021	0.760531	0.788197	0.781007	0.773527	0.779442	0.807741
2W3							1	0.605709	0.892979	0.908558	0.906545	0.880039	0.894734	0.902932	0.880018	0.869591	0.595607
2W4								1	0.50946	0.441372	0.506893	0.457972	0.444409	0.436495	0.412978	0.454206	0.933337
4L1									1	0.97918	0.996025	0.991765	0.936001	0.963231	0.908074	0.908773	0.482403
4L2										1	0.985369	0.97936	0.980258	0.992039	0.964797	0.954639	0.445441
4L3											1	0.993014	0.952263	0.977174	0.929789	0.921749	0.483847
4L4												1	0.937975	0.972046	0.916668	0.898878	0.437149
4W1													1	0.989129	0.99392	0.984846	0.455602
4W2														1	0.979569	0.96028	0.439766
4W3															1	0.976477	0.433885
4W4																1	0.467272
REF																	1

4.4.5 Principal Components Analysis (PCA)

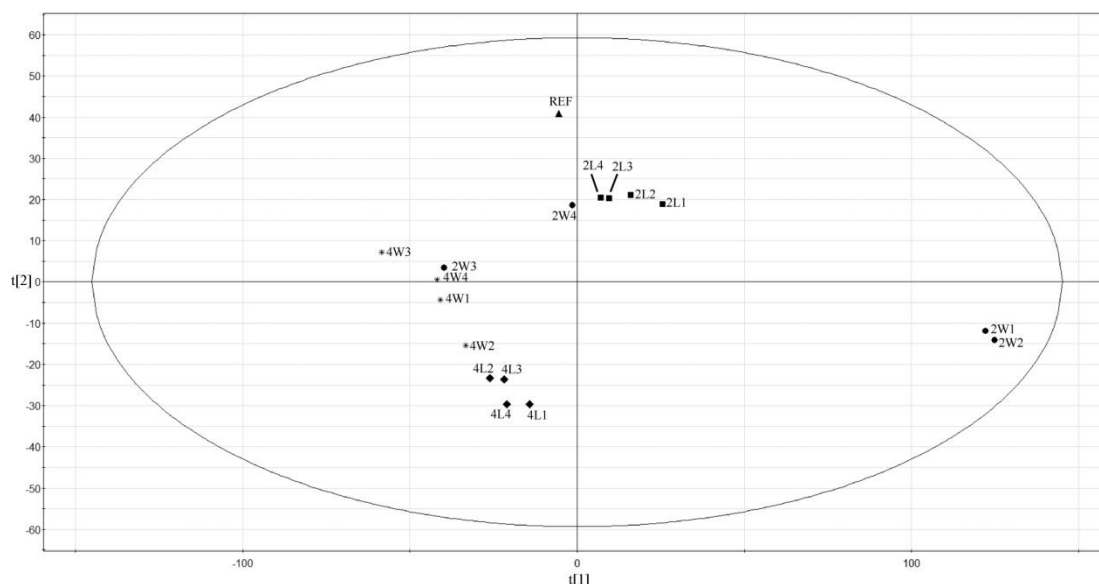


Figure 4.2. Scores plot of PCA of *G. pentaphyllum* samples.

2L1-2L4 represent diploid leaf botanical; 2W1-2W4 represent diploid whole-plant botanical; 4L1-4L4 represent tetraploid leaf botanical; 4W1-4W4 represent tetraploid whole-plant botanical; REF represent reference botanical. Principal component analysis (PCA) transforms a number of possibly correlated variables into a smaller number of uncorrelated variables called principal components, and is a widely accepted mathematical approach for reducing the dimensions of multivariate problems. It can reduce the dimensionality of the original data by introducing small number of underlying factors without losing much information (Chen, Zhu, Xie, Nie, Liu, Li et al., 2008; Kong et al., 2009). PCA analysis has been commonly used for summarizing chromatographic fingerprint data, and may reveal more relationships of the data in a way which better show the variance of the data. **Figure 4.2** shows the PCA score plot prepared using the normalized areas of the 24 characteristic HPLC-UV peaks against the reference peak, the peak 5 at a retention time of 11.21 min

(**Figure 4.1**), while **Figure 4.3** represents the corresponding loading plot. The score plot reflects the original data in a rotated coordinate system, whereas the loading plot shows the weights for each original variable when calculating the principal components. The PCA score plot indicated that the diploid (2L1-2L4) and tetraploid (4L1-4L4) leaf samples and the tetraploid whole-botanical *G. pentaphyllum* samples (4W1-4W4) can be discriminated by their positions, whereas diploid whole-plant samples (2W1-2W4) were dispersed. Also noted was that the diploid leaf samples (2L1-2L4) were close to reference botanical *G. pentaphyllum*, suggesting close relationship and similar flavonoids profile between them, which agreed with visual observation in **Figure 4.1**. In addition, tetraploid whole-botanical samples (4W1-4W4) were closer to their whole-botanical counterparts (4L1-4L4), than to the diploid leaf samples (2L1-2L4). Two of the tested diploid whole-botanical samples, 2W1 and 2W2, were separated from other samples including the other two diploid whole botanicals, 2W3 and 2W4. Interestingly, 2W3 was closer to 4W1-4W4, but 2W4 was closer to 2L1-2L4, suggesting that flavonoid compositions of whole botanicals of diploid *G. pentaphyllum* might be greatly different from each other.

4.4.6 Chemical Markers

Generally, the loading plot of a variable on a principal component (PC) reflects both how much the variable contributed to that PC, and how well that PC takes into account that variable's variation over the data points. Loadings also describe the relationship between variables. So, if the score plot can discriminate the different classes of samples, the loading plot can partly express the influence of

variables on separation between classes. These variables having the greatest influence on the score plot are those further away from the main cluster of variables.

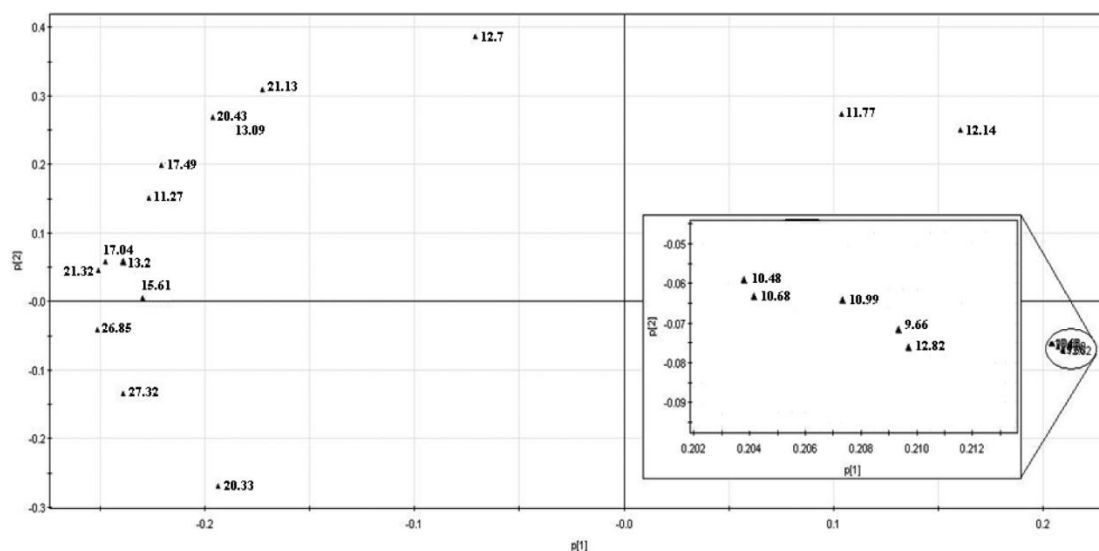


Figure 4.3. Loading plot of PCA of *G. pentaphyllum* Samples.

The rectangle in the bottom right corner is the enlarged graph of the circle. The loading plot of PCA (**Figure 4.3**) indicated that the peaks at retention time 9.66 min (peak 1, quercetin-di-(rhamno)-hexoside), 10.49 min (peak 2, kaempferol 3-O-di-p-coumaroylhexaside), 10.68 min (peak 3, kaempferol 3-O-di-p-coumaroylhexaside), 10.99 min (peak 4, quercetin-rhamno-hexaside) and 12.82 min (peak 9, kaempferol-3-*O*-rutinoside) may have more influence on the discrimination of the samples from different genotypes. It could be seen from **Figure 4.1** that these “chemical markers” existed in tetraploid leaf and whole-botanical samples, not in diploids leaf or whole-plant samples or reference botanical *G. pentaphyllum*, which indicated the chemical profiles of genotypes of diploids were more similar to reference *G. pentaphyllum*.

In summary, this study showed that methanol is a preferred solvent for flavonoids extraction from *Gynostemma pentaphyllum*, and Soxhlet extraction is superior to sonication for high flavonoid yield. The present study reported the rutin and quercetin contents of the leaf and whole-plant tetraploid *Gynostemma pentaphyllum* for the first time, along with comparison with their counterpart diploid samples. PCA analysis of the HPLC-UV fingerprints showed the difference between the diploid and tetraploid *Gynostemma pentaphyllum*, and possibly between leaf and the whole-plant samples from the same genotype of *G. pentaphyllum*, with the diploid leaf samples most similar to the reference sample. These results may imply different physiological activities of the *G. pentaphyllum* diploids and tetraploids, and their potential applications in improving human health.

Summary

The goal of this research was to promote the use of selected jiaogulan to improve human health while enhancing food and agriculture economy. The current investigation discovered that commercial jiaogulan samples differed in chemical composition, and antioxidant, anti-proliferative and anti-inflammatory properties. The genotypes (diploid vs tetraploid) and parts (leaf vs whole-plant) may as well alter nutraceutical properties of jiaogulan products. An HPLC fingerprinting method, combined with chemometrical analysis was developed to differentiate jiaogulan for quality control and quality assurance purposes.

Future investigations of jiaogulan should include a more comprehensive look at the bioactive chemical profiles. Bioavailability and metabolic effect of bioactive compounds in vivo will be needed to better understand jiaogulan's health beneficial effects. In addition, sensory and stability analysis might be conducted while jiaogulan is incorporated into food products.

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